STUDY OF INSULIN ATTACHED ONTO MAGNETIC NANOPARTICLES

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By

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Abstract

Glucose regulation is compromised in diabetic patients and hence diabetes is characterized by accumulation of glucose in blood. As a standard practice diabetic patients usually self-administer subcutaneous insulin injections daily, which are usually associated with pain, tissue necrosis, microbial contamination and nerve damage to local areas. Glucose-responsive implantable devices have provided a hope for a brighter future of diabetes management. However, one of the limitations of such devices is their refill requirement, which often requires surgical procedures leading to lower patient compliance. To overcome this limitation led to the idea of reusing insulin after it has been in the body circulation system and later becomes residues. To make this idea work, the first step proposed in this thesis is to tag insulin with magnetic nanoparticles and then to use a magnetic guidance system to bring it back the residue insulin to the implanted device before it can go to the clearance sites. Obviously, the precondition for the foregoing idea to work is to make sure that insulin's conformation is not affected by the attachment with magnetic nanoparticles. This thesis was designed to study this precondition. The hypothesis is that the insulin's conformation will not be affected by the attachment with the magnetic nanoparticles. Two specific objectives are: (1) assessment of the feasibility of potential capturing techniques and analysis of the attachment of insulin onto the magnetic nanoparticles to confirm the attachment; (2) measurement of the insulin's conformation before and after it is attached with the magnetic nanoparticles. The spectroscopy techniques, including Fourier transform infrared, circular dichroism, absorbance and fluorescence spectroscopy, were used to conduct data collection and analysis. All four of these spectroscopies provide important information concerning the research objectives of this thesis. The results from the fluorescence and absorbance spectroscopy confirm the attachment of insulin onto the magnetic nanoparticles,

hence the achievement of Objective 1. The results from the CD and FTIR spectroscopy show that insulin's conformation is unchanged before and after its attachment onto magnetic nanoparticles, hence the achievement of Objective 2. The general conclusion of the study is that the insulin's conformation will not be affected by the attachment of it with magnetic nanoparticles.

Acknowledgements

First of all I would like to express my sincere gratitude to my supervisor, Professor W.J. (Chris) Zhang and co-supervisor Professor Ildiko Badea for their continuous support and guidance throughout this process. Their inquisitiveness and critical attitude towards research encouraged me further. No words are enough to thank them for their advice and show my appreciation for them. Secondly, I would also like to acknowledge my committee members Chris Zhang, Ildiko Badea, Daniel Chen, and Qiaoqin Yang, and thank them for their valuable suggestions. Without the support and encouragement that I received from my supervisors and committee members I would not have been able to make significant progress.

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List of Abbreviations

AA	**A**mino **A**cid
ATP	**A**dinosine **t**ri**p**hosphate
BG	**B**lood **g**lucose
CD	**C**ircular **D**ichroism
CGM	**C**ontinuous **g**lucose **m**onitors
ConA	**C**oncanavilin **A**
CT	**C**omputed **t**omography
EPR	**E**nhanced **p**ermeation and **r**etention
FTIR	**F**ourier **T**ransform **I**nfrared **S**pectroscopy
IDE	**I**nsulin **d**egrading **e**nzyme
IP	**I**mmuno**p**recipitation
MNP	**M**agnetic **n**anoparticle
MRI	**M**agnetic **r**esonance **i**maging
NP	**N**ano**p**article
PBA	**P**henyl**b**oronic **a**cid
PEG	**P**oly**e**thylene **g**lycol
PEGDA	**P**oly**e**thylene **g**lycol **d**iacrylate
PET	**P**ositron **e**mission **t**omography
RES	**R**eticulo**e**ndothelial **s**ystem
SPION	**S**uper**p**aramagnetic **i**ron **o**xide **n**anoparticles
TCAC	**T**raditional **C**olumn **A**ffinity Chromatography

Chapter 1: Introduction

1.1 Background

The world is rapidly changing due to the climatic changes of the universe, which have resulted in increased hormonal instabilities among the human race. These changes are enhancing the level of uncertain diseases along with aggravating the diseases which are already curable in our medical and scientific realms. It has become a dire need to develop advanced treatments and medical propositions which will allow us to overcome the effects of these overwhelming changes of the environment on humans. Deducing clinically relevant strategies and promoting a healthy lifestyle will provide us with hope to lead a secure life. Diabetes was once considered to be a disease that could be managed easily by adapting our food intake, leading an active lifestyle and taking the required medicine. However, currently diabetes along with obesity has turned into an epidemic in the world and especially in the North American communities.

Diabetes is a chronic condition which affects the body's ability to properly use the energy found in food, usually in the form of glucose. Under normal circumstances, body breaks down the sugars in the food into glucose which then fuels the cells in the body. However, in order to do this, cells require a special hormone called insulin which helps them to take in glucose and use it for energy. Diabetes is a disorder of glucose regulation characterized by the improper accumulation of glucose in the blood. This happens because either our body doesn't make enough insulin or it does produce it but cannot use it. This then leads to the improper accumulation of glucose in the blood. High blood glucose levels can damage the blood vessels in different parts of the body including kidneys, heart, eyes and even the nervous system. Therefore, if left untreated diabetes can lead to heart disease, stroke, problems with eyesight and

nerve damage (http://www.diabetes.ca/diabetes-and-you/complications., accessed February 5, 2018). There are two types of diabetes, Type I (insulin-dependent) and Type II (insulin-independent). Although there is no cure available for both types of diabetes, gastric bypass surgery, lifestyle alteration and medicinal treatment can result in remission. Type I diabetes is usually treated with regular injections of insulin along with controlling blood pressure, cholesterol levels, physical activity and dietary modifications. Type II diabetes on the other hand can be managed with regular use of medication along with exercise, healthy food choices, controlling blood pressure and cholesterol levels (Nichols, 2017).

In both conditions the body transfers itself into a chronic condition which affects the body's ability to regulate glucose levels within the blood. The resulting loss of homeostatic mechanisms leads to fluctuating levels of glucose in the body: either it can go towards a very high scale, which is referred to as Hyperglycemia, or towards the very low end of the scale, which is referred to as Hypoglycemia.

1.2 Motivation

This thesis concerned Type I diabetes, so insulin administration is crucial. Currently, self-administration of insulin multiple times a day via syringes or pens based on an estimated need of individual patients is used alongside frequent monitoring with finger-prick testing. This type of insulin therapy is painful and requires strict self-discipline by the patient, leading to poor compliance. In addition, it can result in periods of hypo- or hyperglycemia with variability in caloric count, meal schedules, metabolism, and exercise ultimately hindering quality of life (De Grauw et al., 2001; Bradley et al., 2002; Yamada et al., 2007). This method of insulin administration is considered to be an open-loop system; it requires an external stimulus, patient

intervention in this case, and fails to mimic physiological conditions, in which the pancreas swiftly releases insulin in response to a rise in blood glucose. Other methods of insulin delivery, such as oral, intranasal, and transdermal have been studied. But, these therapies were also in an open-loop fashion and had limitations regarding bioavailability and clinical efficacy (Lowman et al., 1999; Hinchcliffe et al., 1999; Ma et al., 2006; Chen et al., 2011; Fonte et al., 2013).

The focus, currently, is on methods that involve non-invasive insulin administration which also provides long term release and involves closed-loop based delivery. Closed-loop insulin therapy is a promising avenue of research, because it has the potential to avoid hypo- or hyperglycemic periods, increase compliance, and improve the quality of life in comparison with other therapies by eliminating the need of patient intervention and simulating insulin release by the pancreas (Farmer et al., 2008). Implantable insulin delivery devices with glucose responsive methods have gained a lot of attention. A lot of notable work has been done on these devices, which shows promise with further investigation. They have the potential of freeing the patients of having to monitor their glucose levels continuously. These devices are still under development, and one common problem that was seen in literature regarding these devices had to do with the insulin refill. In most cases, it requires surgical methods, and this causes a low compliance rate among the patients, along with complications that might arise after such a surgery. Reduction of the number of times of insulin refilling is the problem studied by this thesis.

1.2.1 The Big Picture

Our group proposed an idea to address this problem. If the patients could use the insulin stored in the implantable devices for multiple cycles it would increase the availability of insulin and would subsequently decrease the number of times of insulin refilling. Figure 1-1 presents an overview

of this idea. If insulin can be tagged with something like a magnetic nanoparticle or an antibody, it might be possible to pull that insulin back to the implantable device after it has performed its function so that it can be released once again when needed.

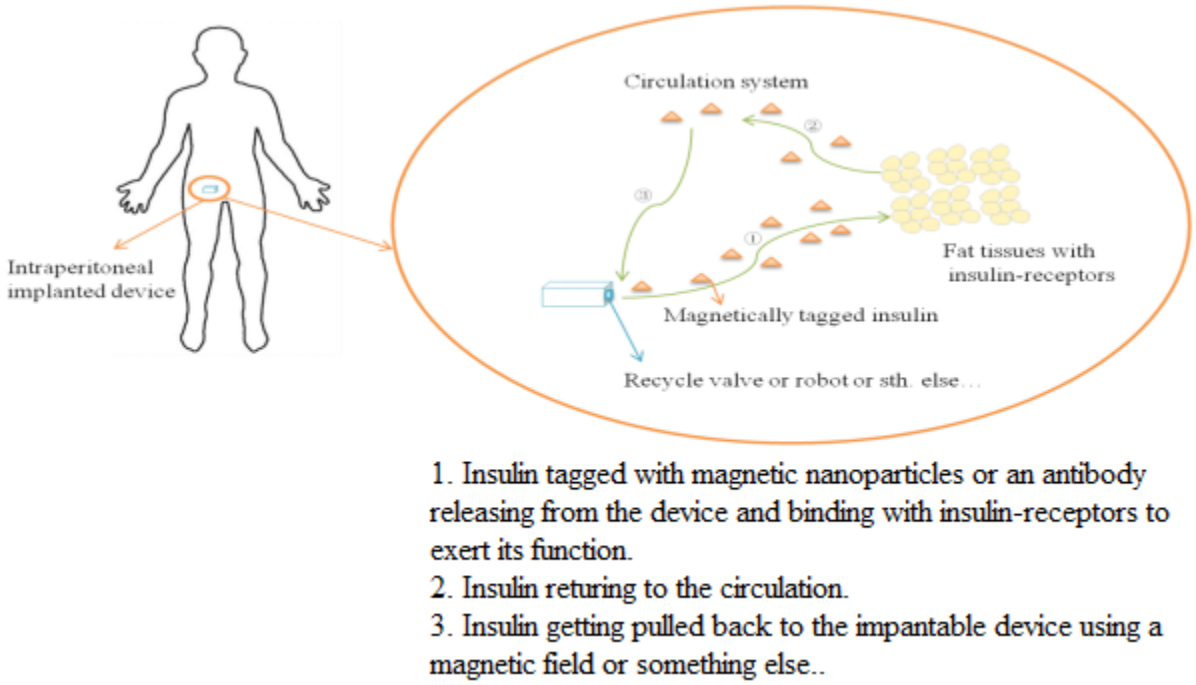

Figure 1-1: The Big Picture

1.3 Research Question

There are two possible options that could be looked at in achieving this goal. The first option is the use of Immunoprecipitation along with an internal or external switch. Specific antibodies could be used to isolate the insulin *in vivo* to protect it from degradation by insulin degrading enzyme (IDE). Although this approach avoids the problems that arise by simply blocking IDE to increase half-life of insulin in the body, this method has drawbacks of its own, which will be presented in detail in literature review in the next chapter. The second approach involves using insulin that is tagged with magnetic nanoparticles. These nanoparticles may then allow insulin to

be pulled back to the device once the insulin dissociates from the insulin receptor. Based on the literature pertinent to the management of diabetes, the capabilities of nanoparticles, and contexts of this thesis study, the second approach was seen to be more viable. Further explanation will be provided in literature analysis in the next chapter.

Based on the above discussion, the following specific research question to this thesis was formulated as: *Can insulin be attached onto magnetic nanoparticles without having its conformation affected by the attachment?*

1.4 Objectives and Scope of the Research

Two specific research objectives were defined for this thesis:

Objective 1: Assessment of the feasibility of potential capturing techniques and analysis of the attachment of insulin onto the magnetic nanoparticles to confirm the attachment.

Objective 2: Measurement of the insulin's conformation before and after it is attached with the magnetic nanoparticles.

Clearly, the achievement of these two objectives will allow generating an answer to the research question of this thesis.

This thesis was restricted to performing *in-vitro* experiments as specified in the statement of the objectives, and the bioactivity testing was not in the scope of the thesis. It involved the literature review to assess the feasibility of potential capturing techniques, experiment to attach insulin onto the magnetic nanoparticles and to perform various characterization experiments to confirm the attachment and to analyze the structure as well as conformation of insulin before and after the

attachment. It is important that insulin retains its native conformation after the attachment because any change in its confirmation might render it inactive going forward. This thesis was also expected to give a comprehensive overview of medical technologies for diabetes, including the medical science behind diabetes, insulin delivery, measurement of glucoses, and so on. This overview is necessary to enhance the understanding of the present work further.

Chapter 2: Literature Review

2.1 Insulin

Insulin is produced by an organ called pancreas. Pancreas has specialized areas called islets of Langerhans, which are made up of different types of cells that release hormones. The most common type of cells found in islets of Langerhans is beta cells, which produce insulin and release it into the blood stream. Beta cells also store insulin while producing it so that it can be released quickly if the need arises. The main function of insulin is to control the use of glucose in our bodies. Glucose is produced when our body breaks down the carbohydrates found in our food. Glucose is the main source of energy for our cells and insulin allows our cells particularly in our muscles, liver and adipose tissue to take glucose up from the blood stream and use glucose to produce energy. Any leftover glucose, not used by cells, is converted into glycogen, and stored as fat for later use, as shown by Step 4 in Figure 2-1 (yourhormones.info., accessed February 5, 2018; Wilcox, 2010).

Insulin has a number of other functions as well and various cell types respond differently to insulin binding onto their surface receptors. Adipose tissue provides a unique example of this; instead of taking up glucose, cells in adipose tissue respond to insulin by taking up the fats in the blood stream and converting them into fatty acids (Wilcox, 2010).

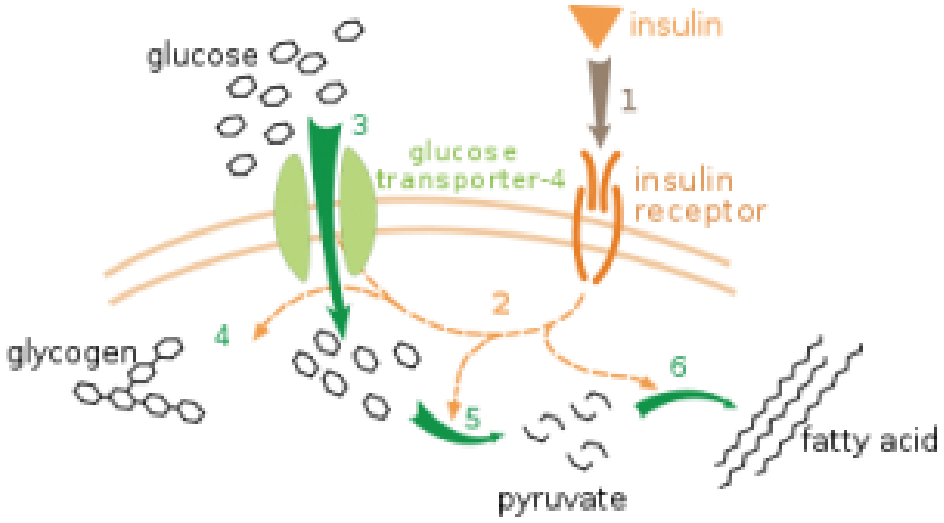

Figure 2-1: Illustration of the insulin and glucose process (Wilcox, 2010). (1) Insulin binds to the insulin receptor, which (2) sends a signal to the glucose transporter, which (3) allows entry of glucose into the cell, then glucose can either (4) be stored as glycogen or (5) be broken into pyruvates, which can then lead to (6) fatty acids. Reproduced from the Nutrition Wonderland website (http://nutritionwonderland.com/2010/05/understanding-our-bodies-insulin/., accessed February 6, 2018).

Besides directly controlling the level of glucose, insulin directs our body on a bigger level to stop digesting fats to produce energy. This leaves glucose in our blood and tissues as the main energy source. Insulin also plays an interesting role in our nervous system by stimulating our body to absorb a lot of its amino acids except tryptophan. This then leads to an increased concentration of tryptophan in blood stream, which makes it easier for tryptophan to cross the blood brain barrier. Tryptophan is used to produce another hormone, serotonin, in the brain, which functions to reduce appetite along with leading to a feeling of happiness. This is associated with the satisfaction that we feel after a good meal. Along with playing a part in regulating our emotions, insulin also has various effects on reproduction, which are not well understood at this time. Few of the examples mentioned above show that insulin is responsible for a lot more along with its

main function of regulating blood sugar levels and is far more important than initially perceived (Wilcox, 2010).

2.1.1 Conformation as it Relates to Insulin

Molecular conformation can be understood as a spatial arrangement of the atoms in a molecule, which can be interconverted. In biopolymers such as insulin the spatial arrangement of substituent groups makes up the molecular conformation. These substituent groups are free to assume different positions in space due to the freedom of bond rotation. Ethane is a relatively simple hydrocarbon and will be used as an example to further explain the concept of conformation. Carbon-carbon single bond in ethane provides nearly complete freedom of rotation around itself. This rotational freedom provides ethane with many different interconvertible conformations based on the angle of rotation. However, if we replace one of the ethane's hydrogens with another functional group with different properties the freedom of rotation will be hindered. This will result in a limited number of stable conformations that this ethane derivative can assume. One of the conformations will be more stable than others and the derivative will assume that conformation. Although insulin is much more complex than ethane, it also abides by the same rules. The different substituent groups on insulin determine its conformation. Changes in the environment such as temperature, pH, phosphorylation or binding of a ligand might induce a change in a biopolymer's conformation (Nature News; Lipid Biosynthesis; accessed September 2018).

As a polypeptide insulin has four levels of structure: primary, secondary, tertiary and quaternary.

Primary: In simple terms, the primary structure of any polypeptide basically displays the amino acids (AAs) that make up the polypeptide in their correct order of linkage. Insulin's primary structure contains two chains: Chain A which is comprised of 21 AAs and Chain B which is

comprised of 30 AAs. In total, the insulin molecule is made up of 51 AAs. AAs in Chain A and Chain B are connected to each other with peptide bonds. At one end of each chain there is an N-terminal with a free amino group ($-NH_2$) and on the other end there is a carboxylic group (-COOH) (https://www.biotopics.co.uk/as/insulinproteinstructure.html.,accessed September 2018).

Secondary: This refers to the next level of structure. The AA chains can form two distinct kinds of structural elements: alpha (α) helices or beta (β) pleated sheets. The third option is for them to link together without any distinct structure. The secondary structure of insulin is dominated by α-helices. Chain A contains two sections of α-helices which have a relatively flat ribbon between them enabling them to essentially lie beside each other. Chain B seems to wrap around Chain A and consists of the larger section of the α-helix. Chain B also has smaller glycine residues which allow it to fold into a "V" shape. This specific folding also allows for some Van der Waals interactions. Any change in the conformation or folding at this level will translate into a change in the higher levels of structure. This is why it is really important to investigate the secondary structure and confirm that polypeptides retain their native conformation at this stage (https://www.biotopics.co.uk/as/insulinproteinstructure.html., accessed September 2018).

Tertiary: This level of insulin's structure involves further stabilization enabled via disulphide bridges. These bridges are formed between cysteine residues each of which has a thiol (-SH) group. Three disulphide bridges are formed in total: two between A and B chains and one within Chain A. At this level salt bridges also start forming and Van der Waals forces become more important as well (https://www.biotopics.co.uk/as/insulinproteinstructure.html., accessed September 2018).

Quaternary: Insulin is usually stored in the form of hexamers (six insulin molecules grouped together). It may also be found in dimers (two insulin molecules grouped together). However, the

active form of insulin consists of a single insulin molecule (https://www.biotopics.co.uk/as/insulinproteinstructure.html., accessed September 2018).

2.2 Insulin Regulation

Although glucose levels are the principal stimulator of insulin secretion; a number of other molecules such as amino acids, proteins, lipids and other hormones like glucagon can similarly stimulate or inhibit insulin release. Also, there is an interesting and marvelous feature of human physiology, which stimulates insulin release even before we start eating. Just being in the presence of food, its smell, appearance, aroma, etc. can cause our brain to start a signaling cascade which triggers insulin release. This is called Cephalic Phase Insulin Release and its function is to prepare our body for what the brain sees as an oncoming flood of glucose. First Phase Insulin Release comes next as the food enters our body. Beta cells release all of the insulin that they had stored in reserves in a burst. The amount of insulin released here depends on how much insulin was needed in response to increased glucose levels that had resulted from a previous meal. If you needed more insulin last time, more will be released and if you needed less insulin, less will be released. Insulin release after this stage depends on whether or not the glucose levels have come back to normal. If more insulin is needed, it will be released otherwise beta cells will slowly take up some of the insulin that was released and the rest of it will go towards insulin degradation pathways (Wilcox 2010).

Another hormone released by pancreas called glucagon has the opposite effect to that of insulin's. Glucagon's job is to raise blood glucose levels while insulin's job is to lower them. This comes into play if blood glucose levels fall too low and there is a risk of hypoglycemia. Insulin and glucagon work in tandem with each other, whereby insulin secretion inhibits

glucagon secretion and vice versa. This feedback system allows our body to tightly regulate glucose levels and make sure that they don't vary much from the homeostatic levels (yourhormones.info., accessed February 5, 2018). This feedback system is represented in Figure 2-2.

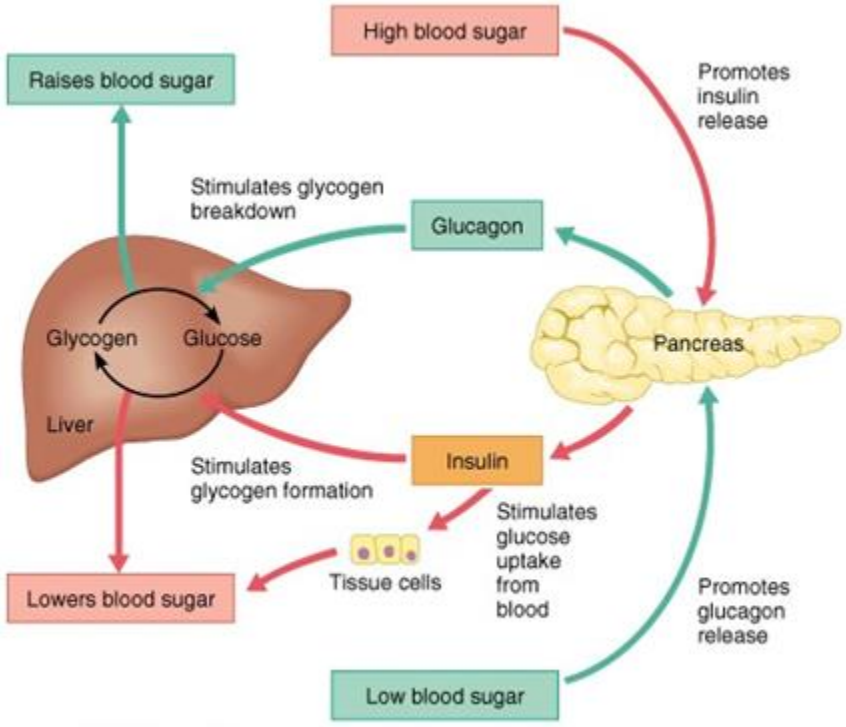

Figure 2-2: Illustration of the insulin-glucose-glucagon feedback system. Chain represented by the red arrows shows what happens when blood glucose levels are high, while the turquoise chain represents what happens when blood glucose levels are low. Reproduced from the Velocity PT Academy website (velocity-pt.co.uk., accessed June 15, 2018).

2.2.1 Glucose Dependent Secretion

Glucose is the primary stimulus for insulin secretion because it is a principal food component in many diets and accumulates quickly after ingesting food. Amount of insulin released in response to glucose versus other substances differs exponentially. Basal plasma level of insulin is 20-30

pmol/L and ingesting 75 g of glucose will raise this to 250-300 pmol/L in 30 minutes, while ingesting the same amount of fat and protein will only raise it to 50-60 pmol/L in humans (Chang et al., 1978). A number of evolutionary adaptions allow beta cells to continuously monitor the plasma glucose levels. These adaptions are coupled to rapid oxidative and anaplerotic metabolism which basically helps translate the signal resulting from increased glucose levels and maximizes production of adenosine triphosphate (ATP) which can be used for insulin secretion. Within the physiological range, beta cells are able to sense glucose because of their high K_m glucose transporters and glucokinase. A high K_m means that these transporters and glucokinase have lower affinity for glucose and have a smaller residence time at the binding site which results in higher turnover. Lower expression of lactate dehydrogenase, higher expression of redox shuttles, resulting in regeneration of reducing equivalents and increased pyruvate dehydrogenase and pyruvate carboxylase activity, also contribute towards having an efficient oxidative metabolism while glucose is present in abundance (Newsholme et al., 2012). In short, glucose impacts insulin secretion in beta cells via number of ways, with ATP generation and enhancement of oxidative metabolism being central to the process.

2.2.2 Amino Acid Dependent Secretion

Amino acid metabolism plays a major role in a number of cellular functions with protein and nucleotide synthesis being the most important. However, if administered as a nutrient source, they also have the ability to regulate insulin secretion. Effects of amino acids on insulin secretion are greatly dependent upon the type of amino acids administered, duration of exposure and concentration. Amino acids need to be administered in specific combinations in order for them to illicit a response. Under such circumstances, amino acids have the ability to regulate the triggering and amplification pathways of insulin secretion in a number of ways. Firstly, they can

serve as substrates for the oxidative cycle and redox shuttles resulting in ATP generation. Secondly, they can directly depolarize the cell membrane by transporting positively charged amino acids into the cell. Finally, they can also act as a co-transporter of sodium ions (Na^+) once again resulting in depolarization of the membrane (Newsholme et al., 2012).

2.2.3 Lipid Dependent Secretion

Lipids also play a crucial role in beta cell function and insulin release; continuously increased lipid levels usually result in dyslipidemia and insulin resistance. Insulin resistance results in an increased need for insulin. This need is compensated by fatty acid potentiated insulin secretion. Also, during periods of starvation or fasting, lipids get metabolized in mitochondria of various cell types to produce ATP. The resulting energy from ATP can be used for insulin secretion (Chang et al., 1978; Newsholme et al., 2012).

2.3 Insulin Degradation

As it is apparent from its definition, the function of insulin clearance is to remove and inactivate the insulin in circulation after it has performed its action. A theoretical approach will dictate that insulin (or any other hormone) degradation is just as important as its secretion. All insulin-sensitive cells and tissues have the ability to uptake and degrade insulin (Castillo et al., 1994). Although insulin clearance is a very complex process with multiple pathways, Insulin Degrading Enzyme (IDE) is thought to be the major enzyme driving this process (Duckworth et al., 1998). Insulin has a relatively short half-life of 7.5 – 9.5 minutes which is expected due to the nature of insulin's function and role as a hormone (Burt et al., 1974). Insulin clearance takes place in the liver, kidneys, extracellular environment and via cellular uptake of insulin and subsequent degradation.

2.3.1 Liver

Liver is a major site of insulin clearance. The mean residence time of insulin is 71 minutes, out of which 62 minutes are spent bound to receptors in the liver that translates to about 80% of insulin being bound to liver receptors (Hovorka et al., 1993). Hepatic clearance of insulin is often affected by physiological and pathophysiological factors. For example, glucose ingestion results in increased insulin secretion which translates to increases in hepatic insulin uptake and decreases in patients with obesity and diabetes. Hepatic uptake and subsequent degradation of insulin relies mainly on a receptor-mediated process (Duckworth, 1988). However, during periods of high insulin concentration pinocytosis (non-receptor-mediated insulin uptake) also plays a significant role in uptake of insulin (Harada et al., 1992).

2.3.2 Kidneys

While liver plays a major role in clearing portal insulin, kidneys are the major players in clearing peripheral insulin from systemic circulation. Kidneys also remove about half of the circulating proinsulin (precursor of insulin) and also play a role in clearing a number of other insulin analogs (Rabkin et al., 1984; Kruse et al., 1997). Kidneys employ two mechanisms for insulin clearance: glomerular filtration and proximal tubular reabsorption. Glomerular clearance can occur via receptor-mediated transport or nonspecific diffusion. Proximal tubule cells in tubule lumen reabsorb almost all of the passing insulin via endocytosis (Nielsen, 1992).

2.3.3 Cellular Insulin Uptake

Cellular insulin uptake is a typical biological method with a number of steps. It starts with insulin binding onto to the receptor. This receptor bound insulin can then either be returned back

to the circulation intact or it can go to an intracellular site for degradation. Furthermore, there are multiple pathways for degradation of internalized insulin, which involve degradation in endocytotic vesicles. Degradation products can go to multiple subcellular sites such as the nucleus, golgi or even cytosol. Degradation can either take place at these sites or insulin is transferred to lysosomes. Most of the internalized insulin and its degradation products end up in lysosomes in order for degradation to be completed (Duckworth et al., 1998). Figure 2-3 shows a model of this process.

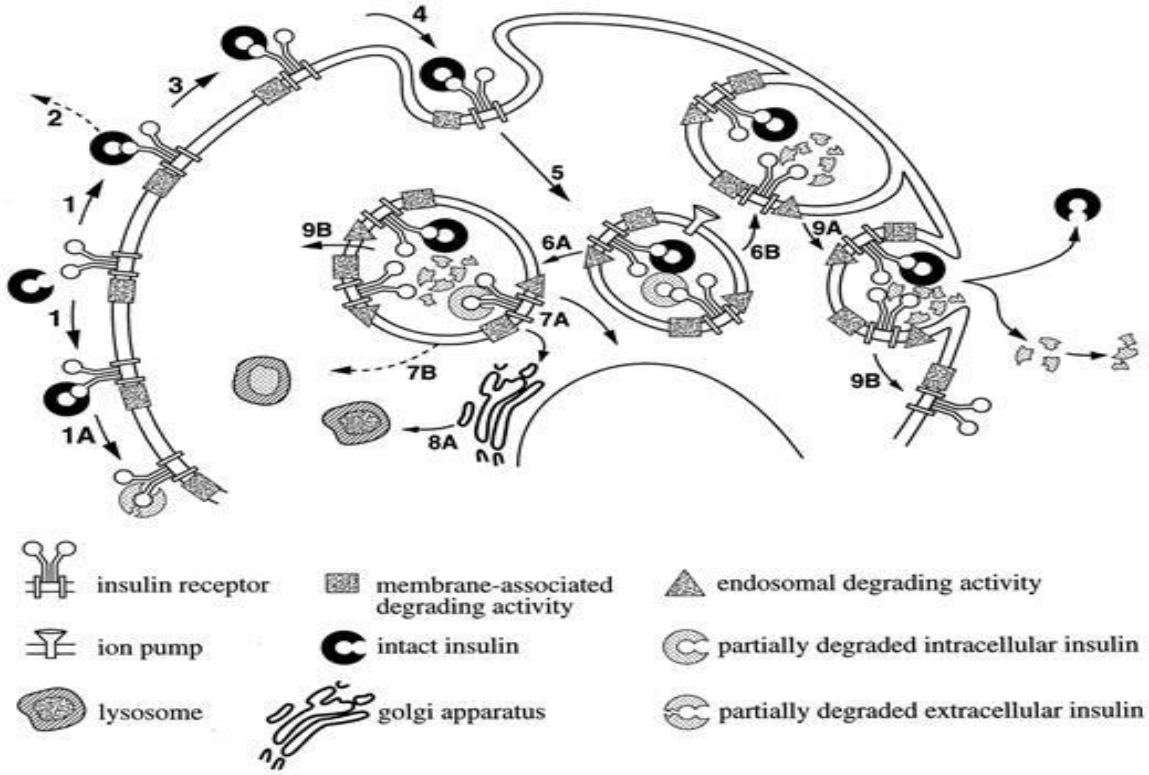

Figure 2-3: Illustration of the cellular handling and degradation of insulin. (1) Insulin binds to its receptor; (1A) represents degradation right on the membrane; (2) possible release of intact insulin; (3) co-localization of insulin in plasma membrane; (4) formation of coated pits; (5) endocytosis; (6) initiation of endosomal degradation; (6A) intracellular processing of insulin and its degraded components;(6B) diacytosis of intact insulin or its degraded products; (7)

insulin or its degraded products are distributed intracellularly; (7A) cytoplasm, nucleus, golgi; (7B) lysosomes; (8) insulin delivery to lysosomes from organelles besides endosomes; (9) insulin receptor getting recycled; (9A) delivery of the receptor to the plasma membrane by diacytosis; (9B) insulin receptor back in its initial state. Reproduced with permission from W. C. Duckworth: Endocr Rev 9:319–345, 1988 (2). ©

2.4 Glucose Responsive Implantable Device

As mentioned earlier exogenous insulin therapy is the main method employed by patients to manage their diabetes. However, there are various compliance issues associated with this method and more importantly it does not provide an accurate required dosage which can lead to poor clinical outcomes. Blood glucose levels cannot be predicted accurately mainly because of the variations in meals and body metabolism which can often lead to a higher or lower dosage response than is expected. Mostly the patients present with hypoglycemia (low blood sugar) due to an increased effect of the dosage. Glucose Responsive Implantable Devices have the potential to get rid of these limitations. They will provide an accurate release of insulin based on the close-loop delivery principle. Glucose responsive implantable devices are a major step forward from the outside pumps that had been the center of attention before. Unlike an outside pump which detects the levels of glucose from the skin which subsequently results in a delay, these devices release insulin by detecting the actual blood glucose level within the body. Glucose levels are continuously monitored and based on the changes either the insulin release is initiated or it is inhibited or slowed down. In short, they provide closed-loop insulin delivery based on the glucose levels. Figure 2-4 depicts how these devices work in principle.

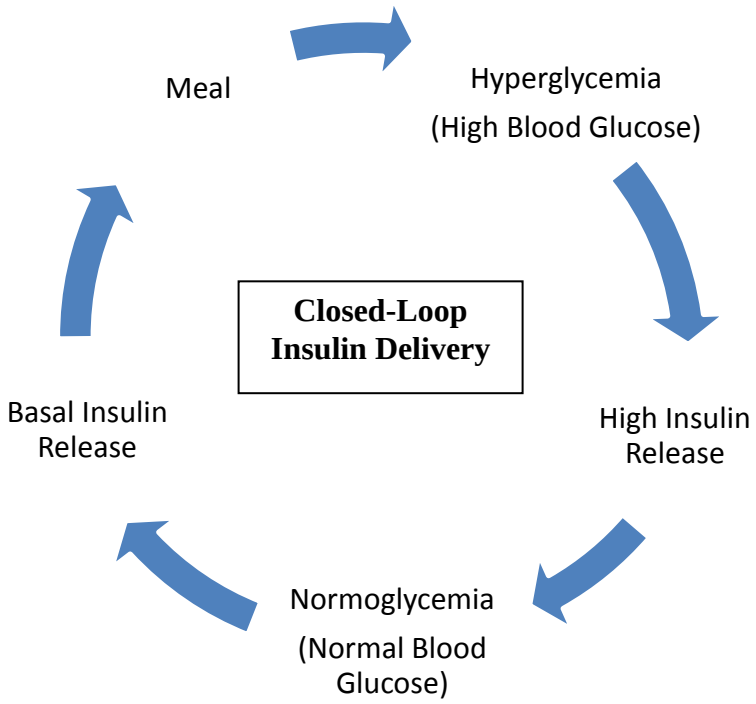

Figure 2-4: Illustration of the closed-loop insulin delivery principle.

Weller et al. (1960) was the first to discover the fact that artificial pancreas could be used as a tool to save the effort of injecting patients at various intervals. Learning from Weller's research, Kadish in 1964 worked on developing the first portable insulin device which was actually more like a pumping machine. Although this system had a lot of limitations such as the absence of accurate computational aid to release insulin, it is still recognized as the first approach towards a closed-loop glucose responsive system. Weller's research and Kadish's initial attempt acted as a flame of fire to the oil in the realms of medical sciences which proved to be a door towards a whole world full of innovative techniques. Since then, many portable devices for glucose detection and acceptance have been designed and implemented to satisfy the thirst of gaining efficient clinical results. In 1970s Albisser et al. and Pfeiffer et al. took another step forward and navigated the world towards closed-loop delivery device. Both of these groups, independent of each other, developed a true artificial endocrine pancreas. They combined the continuous glucose

monitors with computational aids, a microcomputer with algorithms imbedded in it to automate the release of insulin. Further developments were made in the next two decades to make the devices more efficient and patient friendly. A number of algorithms were developed in hopes of mimicking the physiological secretion and inhibition of insulin. However, better methods were still needed because human body's responses in terms of metabolism cannot be predicted accurately by such an algorithm (Hovorka, 2006).

Recently, research has been directed into overcoming the inadequacies of the previous devices through development of subcutaneous implant systems. In 2015 Chu et al. performed surface modification on their previous device using high molecular weight polyethylene glycol (PEG) and subcutaneously implanted it in rats to achieve a 3-fold increase in *in vivo* efficacy. Similarly, another group used subcutaneously implanted membranes constructed from a biocompatible polymer modified with a PEG monolayer and followed specific implant geometry and surface topography to achieve a functional lifetime of 21 days of their device (Li et al., 2015). However, a major shortcoming of subcutaneous systems is that they lack in speed and stability of insulin delivery in comparison to the physiological system. Intraperitoneal insulin delivery has faster kinetics and better stability and reliability than the subcutaneous route (Burnett et al., 2014; Renard, 2008; Renard et al., 2011). Thus, Taylor et al. tested an intraperitoneal implant in a domestic pig with induced diabetes and were able to achieve normoglycemia for about a month. The pig was terminated before the insulin was refilled due to a peritoneal infection (Taylor et al., 2016). Thus, ways to prolong implant functionality in the intraperitoneal cavity should be investigated in order to minimize invasive procedures for replacement/refilling of insulin reservoirs while maintaining the speed and reliability of intraperitoneal insulin delivery systems.

2.5 Review of Potential Capturing Techniques

2.5.1 Immunoprecipitation

Immunoprecipitation was developed as the next step in the Traditional Column Affinity Chromatography (TCAC). TCAC used a packed column of porous resin which had target-specific antibodies attached onto it. The samples would then pass over this resin and the immobilized antibody would form a complex with its antigen (target proteins or biomolecules). However, IP doesn't rely on a packed column and it involves a number of incubation steps and uses only a small amount of resins in a microcentrifuge tube. Immunoprecipitation uses an antibody which specifically binds to an antigen and precipitates it out of the solution of many antigens. The most widely used application of IP is the small-scale affinity purification of antigens using specific antibodies which are immobilized onto solid supports such as an agarose resin or magnetic particles. Although a variation of IP called co-immunoprecipitation can be used to study the protein-protein interactions, in its simplest form, and for our purposes as well, IP is used to isolate a single antigen.

IP assays can be performed in one of the two ways: either by using a pre-immobilized antibody on a highly porous agarose or solid magnetic beads which are then incubated with the solution containing the antigen or by using non-bound antibody which can form complexes with the antigen which are then retrieved by the beads. As Figure 2-5 depicts, the result using both methods would be same. Up until the recent past, majority of the IPs were performed using agarose beads, but now the use of magnetic beads has become more common. The reason for it is the ease of use, reproducibility, increased purity and speed. Overall, each method has its benefits and drawbacks and the final decision is ultimately based on the target protein and the type of IP assay to be used (thermofisher.com., accessed February 15, 2018).

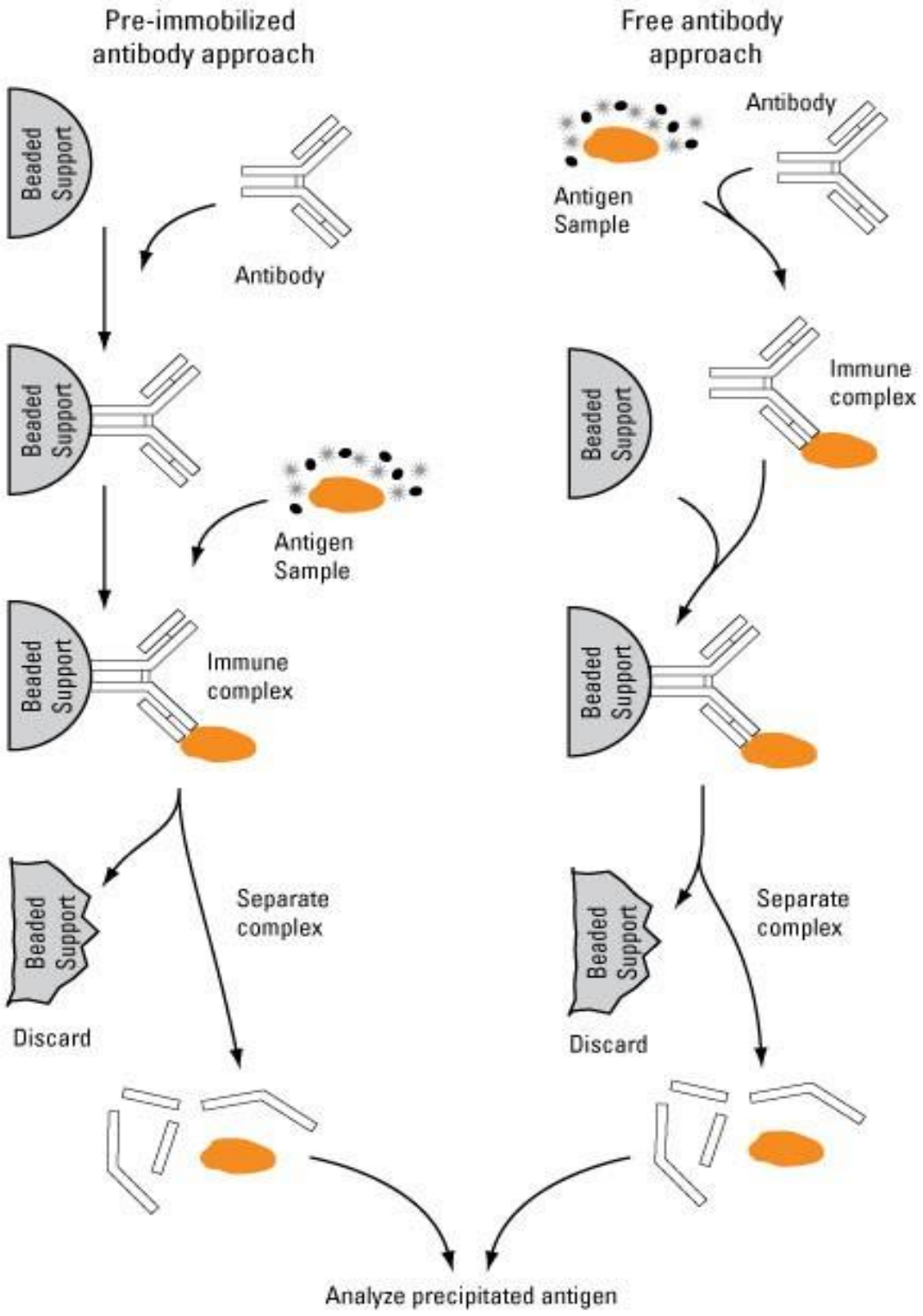

Figure 2-5: Illustration of immunoprecipitation using pre-immobilized (left) and free (right) antibodies (thermofisher.com*., accessed February 15, 2018).*

Magnetic Beads versus Agarose Resin

Initially IPs were performed using small aliquots (10-25 µL) of agarose resin in microcentrifuge tubes. After the incubations, centrifugation is used to separate the agarose beads from solution. The easiest way to do this is to use a microcentrifuge filter cup which retains the beads and collects rest of the solution in the tube. A more time consuming technique involves pelleting the beads and carefully pipetting off the solution. These characteristics of agarose resin prevent it from being used in experiments which require significant automation or scaling down. These limitations have led the agarose resin to be replaced by magnetic particles such as Dynabeads or Pierce Magnetic Beads. Antibodies can only bind to the surface of each solid and spherical magnetic particle. Magnetic beads make up for this limitation because of their significantly smaller size (1-4 µm) compared to agarose beads (50-150 µm), which collectively give them increased surface area. High power magnets are then used to localize the beads to the side and rest of solution can easily be poured or pipetted out. This eliminates the need for centrifugation which can break off relatively weak interactions between antibody-antigen resulting in loss of target antigen. This process can be fully automated (thermofisher.com., accessed February 15, 2018).

As mentioned before, IP techniques are mainly used to isolate a certain antigen in-vitro for further analysis. It will be relatively easy to isolate insulin from a cell or tissue lysate using the IP techniques. However, for our purposes this is where IP use is limited to, and we need further capabilities.

Magnetic Beads

For the purpose of this research, an insulin specific antibody would have been used, which would help with isolating the insulin, so insulin won't be degraded, and then later collected to be released once again when needed. The collection part suggests that using the superparamagnetic beads would be a better option in the experiment, since the insulin-antibody complex would need to be pulled back to the insulin device and a magnetic field could be used to achieve this. Although in theory this approach seems to be sound, its limitations prevent us from using this technique. First of all, magnetic beads are only recommended for small sample sizes of about 2mL, which suggests some doubts regarding its use in the circulating blood of much greater volume. Second, the use of magnetic beads in IP requires the use of high power magnetic equipment which can prove to be quite cost prohibitive. Also, this process requires the process conditions or environment to be very specific in terms of the required pH, temperature, bead size, etc. Any variations in these parameters could prevent the beads from localizing onto the magnet. For a long term use, magnetic beads coated with antibodies need to be stored at a temperature range of 2^{o} - 8^{o} C, otherwise they will lose the antigen binding capacity (thermofisher.com., accessed February 15, 2018). It is virtually impossible to mimic such conditions inside a human body for long periods without significantly affecting other functions of the body.

Agarose Resin

Using this technique only gives us the opportunity to isolate insulin and prevent it from degradation. There is no feasible approach which allows the insulin from the agarose resin to be pulled back to the implanted device. Gel bed like agarose resin structures will need to be placed at various insulin clearance sites so that they can capture insulin before it gets to liver or kidneys

for degradation. Agarose resin does allow us to work with a larger sample size, however accessibility to antibodies is sometimes diminished in these resins because of their sponge like structure. This approach also requires longer incubation periods, and this might not be possible due to insulin's bioavailability after it has affected its action. This technique also requires very specific conditions to work efficiently (thermofisher.com., accessed February 15, 2018).

Main reasons for not using IP

The use of immunoprecipitation is highly dependent upon the specificity of the anti-insulin antibody used. This rules out the use of polyclonal antibodies which can bind to different sites on the same antigen. They might bind to other protein complexes which might have the same epitope in their long amino acid chains. A significantly more expensive monoclonal antibody will need to be produced, which will actually have a lower affinity for insulin as it can only recognize one site or epitope on insulin. Due to this limitation, monoclonal antibodies require longer periods of time to capture the target protein, which as mentioned before might not be feasible due to insulin's relatively short bioavailability. Monoclonal antibodies are also more sensitive to changes in their environment, and this might render them unable to detect insulin (pacificimmunology.com., accessed February 15, 2018). The biggest reasons lie within the processes that might be required to dissociate insulin from the insulin-antibody complex so it can be used again. Currently no gentle methods are available to achieve this dissociation; indeed, extreme pH and temperature changes or the use of toxic chemicals is required to achieve this. Since the long term approach for this idea is to be used *in vivo*, implementing these extreme changes or the use of toxic chemicals will not be feasible as they will interfere with rest of the homeostatic systemic circulation as well. The dissociation process also does not guarantee the

functionality of insulin or the antibody after dissociation as in most cases it results in denaturation of the antibody and antigen.

Our research group was collaborating with abcam, a company that specializes in antibodies and their use in various techniques including IP, to look for specific antibodies that would comply with our requirements. A lot of their polyclonal antibodies were ruled out based on our requirement of specificity. For any of their polyclonal or monoclonal antibodies, currently, no protocol is available that will allow us to dissociate insulin from the antibody. An extensive trial and error would be required to find a suitable dissociation procedure, which might still not be feasible to use *in vivo*. In literature, there are conflicting views regarding using the same antibody more than once and antibodies available at abcam will once again require extensive trial and error to figure out whether or not they can be reused and how many times they can be reused. Based on the extensive amount of extra work and resources required to consider and address various complications and variations that might arise using IP our research group decided it to not be a feasible approach for our purposes.

2.5.2 Nanomedicine in Diabetes Management (Magnetically Charged Nanoparticles)

Over the past few decades, starting with the FDA approved application in cancer treatment (Barenholz, 1995), incorporation of nanotechnology in novel diagnostic and treatment strategies in various medical fields has improved their efficacy immensely. Nanotechnology's successful employment in cancer treatment and various other diseases including cardiovascular diseases lit an ever spreading fire in the realm of medicine. Researchers found the chemical, physical and biological properties of materials produced from nanotechnology, such as nanoparticles, very tempting for use in medicine and numerous other biomedical applications (Whitesides, 2003; Lavan et al., 2002). Since then a lot of nanoparticle formulations have been produced for use in

medicine, including magnetic or polymer nanoparticles, stimuli responsive nanoparticles and various other nanostructures including nanofabricated devices (Kudr et al., 2017). Appreciation for these products of nanotechnology has found its way in diabetes management as well. Current developments include noninvasive monitoring of disease progression and blood glucose levels, glucose responsive insulin therapy and improvements in immune modulation for cell based therapies (Veiseh et al., 2014). Nanomedicine based approaches provide immense potential for improving the management of diabetes in patients with Type I or Type II Diabetes.

Applications in Diagnosis and Disease Monitoring

Research indicates that earlier diagnosis and treatment of diabetes may be beneficial to disease management (Simmons et al., 2010). Pancreatic β-cell mass is an indicator of disease progression and will facilitate an understanding of the development and course of diabetes, as there is a reduction in the cell mass with progression of diabetes (Matveyenko et al., 2008). However, β-cell mass cannot be directly measured *in vivo* in humans. Currently, downstream measures of β-cell mass are used, such as C-peptide levels or insulin requirements. Autopsy studies are required for direct measurement of β-cell mass, which are post-mortem and confounded by several factors such as limited dissection of the pancreas (usually only the pancreatic tail) and changes in β-cell mass associated with the final illness leading to death (Fogar et al., 1994; Chari et al., 2005).

Advancements in molecular imaging have made β-cell mass measurements *in vivo* possible through development of β-cell-targeting peptide dyes (Reiner et al., 2011) and antibody-dye conjugates (Moore et al., 2001), but these techniques require invasive procedures to access tissue samples. Several noninvasive imaging techniques for the purpose of β-cell mass measurement are being investigated, such as computed tomography (CT), positron emission tomography

(PET), magnetic resonance imaging (MRI) (Andralojc et al., 2012). In addition, magnetic nanoparticle (MNP) probes with β-cell specificity that act as contrast agents have been developed to facilitate β-cell imaging (Medarova et al., 2009; Wu et al., 2010). Specifically, superparamagnetic iron oxide nanoparticles (SPIONs) can aid visualization of β-cells via MRI. In a small clinical trial, patients with recent-onset type 1 diabetes were infused with a SPION-based contrast agent called ferumoxtran-10. MRI of SPIONs in these patients allowed visualization of the pancreas and revealed differences between patients with recent-onset diabetes and healthy controls associated with islet inflammation (Gaglia et al., 2011). This technology was exploited as a prediction tool in a future study to achieve early diagnosis of diabetes in young mice through detection of immune cell filtration followed by pancreatic-islet inflammation (Fu et al., 2012). It has further potential to monitor the progression of diabetes and assess effectiveness of experimental therapies early in the trial. The early response to an immunomodulatory intervention was monitored using this technology in diabetic mice (Turvey et al., 2005).

Though these advancements in pancreatic imaging are promising for diabetes diagnosis and monitoring of disease progression, further research is required to develop biomarkers that report β-cell stress or dysfunction. This will facilitate assessment of the effectiveness of therapies and inform categorization of patients by disease severity for targeted therapies.

Applications in Glucose Sensors

Blood glucose levels allow monitoring of the disease to inform therapies as diabetes progresses and predict complications associated with diabetes. Therefore, highly sensitive glucose biosensors that monitor BG levels would be beneficial to fundamental diabetes research.

Currently, handheld glucose meters that use blood samples collected via finger pricks are most commonly used to measure BG levels (Schmid et al., 2013). However, this method is painful, permits only intermittent testing, and makes it possible to miss episodes of hyper- and hypoglycemia that occur outside of the testing times. Therefore, subcutaneously implanted amperometric sensors called continuous glucose monitors (CGM) that sample interstitial fluid have been developed (Mauras et al., 2013). Though CGMs allow for continuous testing, issues remain with accuracy and usability; CGM measurements lag 5-15 minutes behind BG levels, CGM implantation requires an invasive procedure, and the CGMs need to be replaced frequently (Hovorka et al., 2013). Research has been directed to develop glucose sensors that can overcome the shortcomings of the conventional glucometers and CGMs.

For instance, amperometric glucose sensors went through multiple generations of improvement. The first generation of glucose sensors employ oxygen as an electron mediator between glucose oxidase and an electrode, where glucose oxidase enzymatically reduces oxygen into hydrogen peroxide in the presence of glucose (Hovorka et al., 2013). The rate of this reaction is proportional to the glucose concentration. Though a continuous readout of glucose concentration is possible with the first generation sensors, they are dependent on oxygen and may be interfered by competing redox-active species. The second generation sensors overcame the oxygen-dependency by using artificial mediators such as ferro/ferricyanide but still risked interference by other redox-active species that may be present (Cass et al., 1984). The third generation sensors were based on electron transfer directly between glucose oxidase and the electrode, proposed by Heller and Degani (Degani et al., 1987; Heller, 1990), because it eliminated the possibility of interference.

Next, nanosensors with glucose oxidase built onto surface of metallic nanoparticles (palladium, gold, and platinum) and carbon nanotubes were developed because they have a large specific surface area and improved biocompatibility (Zhang et al., 2005; Tang et al., 2004; Claussen et al., 2010). Though these sensors were an improvement, inherent drawbacks associated with glucose oxidase remained; glucose oxidase activity can diminish over time (Hoedemaekers et al., 2008) and be affected by changes in oxygen level, pH, and temperature (Bankar et al., 2009). These limitations led to investigation of nonenzymatic nanosensors. For instance, sensors relying on metal oxide catalysts such as copper oxide (Bankar et al., 2009; Pickup et al., 2005) or gold nanoparticles (Hussain et al., 2011) to oxidise glucose have been developed. These sensors use applied voltage to drive reactions and thus require batteries, making them bulky and inconvenient for patients. Sensors using glucose-binding moieties, including lectins (Schultz et al., 1982; Liao et al., 2008); synthetic molecules such as phenylboronic acid (PBA) (Chen et al., 2011; Kataoka et al., 1995); and polyacrylamide- (Yang et al., 2011) and polyallylamine-based (Yang et al., 2011; Parmpi et al., 2004) hydrogels have also been explored. Nanoparticles such as carbon nanotubes (Barone et al., 2009; Yum et al., 2013) or nano-optodes (Balaconis et al., 2011; Billingsley et al., 2010) in association with the glucose-binding moieties may be able to represent glucose binding to the moiety as a voltammetric or fluorescent output. Single-walled carbon nanotubes and semiconducting quantum dots have been shown to produce a fluorescent output in such sensors (Bull et al., 2012; Klonoff et al., 2012). Such sensors are patient-friendly, as they do not require batteries and thereby may have higher longevity in vivo. They are also more promising than previously mentioned sensors because they do not rely on an enzyme-based catalyst and have the potential to provide rapid readouts.

Despite these advantages, further research is required to develop nanosensors with fluorescent or voltammetric outputs for clinical use. The safety of carbon nanotubes is questionable (Saito et al., 2014), device biocompatibility must be achieved, and calibration of fluorescent output needs to be accomplished across variations in skin such as color and hair thickness.

Applications in Insulin Delivery

Nanotechnology offers unique advantages in insulin replacement therapy by improving its efficacy, safety and ease of use which results in greater patient compliance (Krol et al., 2012; Zhi et al., 2013). Research has also been focused on developing long-acting nanoparticulate insulin formulations to minimize the frequency of injections (Peng et al., 2012) while other research has focused on even more novel ideas that can possibly lead to development of less invasive routes of insulin delivery. As mentioned above, ever since the production of the first glucose-responsive insulin system, the interest in developing glucose-responsive insulin formulations that incorporate nanotechnology has increased immensely. Along with increasing the reliability of glucose level detection the glucose sensors mentioned above can also be incorporated into insulin delivery systems making them glucose-responsive.

The biggest advantage that the glucose-responsive insulin delivery systems provide is the ability to much more closely mimic the physiological insulin cycle in response to changes in blood glucose levels. This will lead to tighter glycaemic control and will result in decreased hypoglycaemic periods. Nanotechnology pertaining to insulin delivery systems has matured very rapidly resulting in advancements that allow researchers to develop insulin carrying nanoparticle formulations that can respond to changes in their environment and subsequently release their cargo (Fleige et al., 2012). There are multiple ways by which a nanoparticle can be made to be

sensitive to the fluctuating glucose levels in its environment. The most commonly used approach involves the use of glucose-sensing triggers such as glucose oxidase, glucose-binding proteins or any other glucose-binding small molecules. Nanoparticle formulations using these triggers respond to increased glucose levels through swelling, degradation or disassembly which results in insulin release (Stuart et al., 2010; Wu et al., 2010). One of the ways that glucose-sensitive nanoparticles can be engineered is by combining glucose imprinted polymers with glucose-binding proteins such as concanavilin A (ConA) to form supramolecular assemblies that are glucose-responsive (Yin et al., 2014). Figure 2-6 below shows how glucose-sensing triggers such as ConA can be integrated into the nanoparticle that is engineered to swell or degrade in response to increased glucose levels.

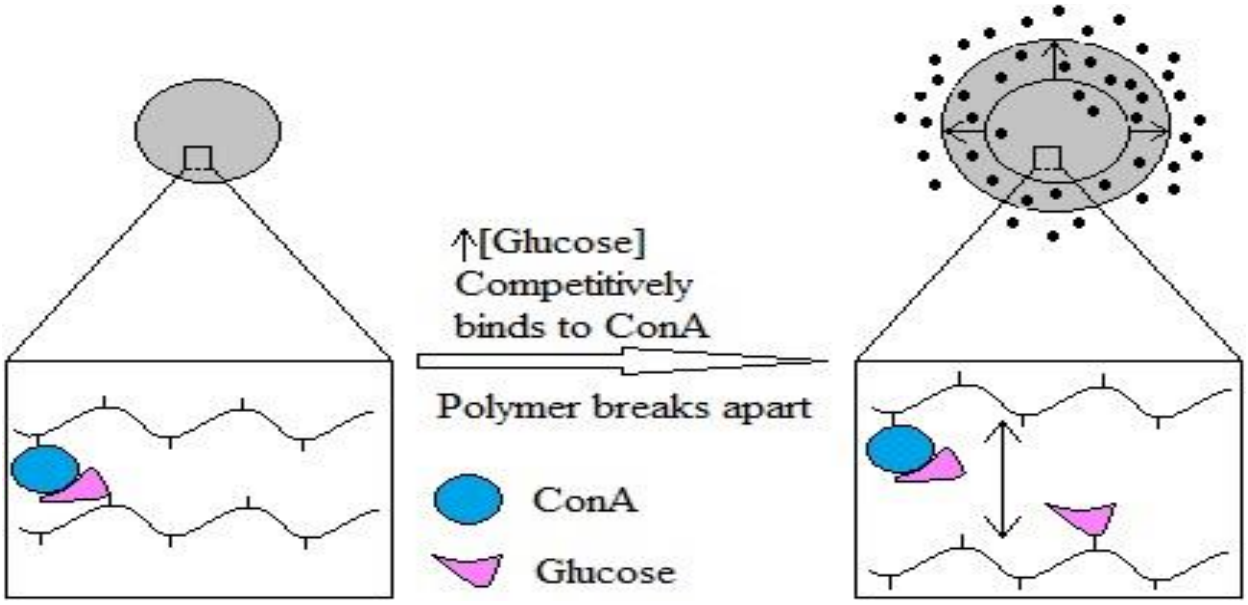

Figure 2-6: Illustration of glucose-imprinted polymer combined with concanavilin A (ConA) to form a supramolecular assembly which is responsive to glucose (Veiseh et al., 2014).

Glucose-binding proteins such as ConA have high specificity for glucose and provide reliable binding. However, this approach has a drawback, glucose-binding proteins can be seen as foreign entities by the body and limited progress has been made in trying to get rid of this limitation.

Perhaps because of this reason most of the research has focused on the approaches based on glucose oxidase and in part due to its high specificity for glucose (Veiseh et al., 2010).

Magnetically Charged Nanoparticles

Magnetically charged nanoparticles (MNPs) are a class of non-invasive therapeutic agents that can be used for imaging, cellular-specific targeting and drug delivery. Recent advances in this realm have made them irresistible for use in various biomedical and therapeutic applications. One of the main reasons for their irresistibility is their intrinsic magnetic properties. MNPs comprise of nanoparticles that make use of their metallic, bimetallic or superparamagnetic iron oxide (SPIONs) cores (Sun et al., 2008; Mcarthy et al., 2008). SPIONs have garnered most of the attention due to their relatively lower toxicity (Lewinski et al., 2008; Wang et al., 2001; Lawrence, 1998) and more importantly their reactive surfaces which can be modified with biocompatible coatings (Gupta et al., 2005; Gupta et al., 2007; Laurent et al., 2008; Misra, 2008) combined with imaging, targeting and therapeutic molecules (Laurent et al., 2008; Misra, 2008; McCarthy et al., 2007; Dobson., 2006). These properties have allowed successful SPION usage in magnetic separation (Pankhurst et al., 2003), biosensors (Zhao et al., 2006; Perez et al., 2002), medical imaging (Sun et al., 2008; Frullano et al., 2007; Corot et al., 2006), drug delivery (Dobson, 2006; Duran et al., 2008), tissue repair (Solanki et al., 2008) and hyperthermia (Thiesen et al., 2008) applications. A few of these SPIONs are already in early clinical trials or experimental study stages (Sun et al., 2008; Mcarthy et al., 2008; Laurent et al., 2008) and some have even been approved for clinical use in medical imaging and therapeutic applications. In its most simple form a MNP consists of a magnetically active core which is coated with a stabilizing shell. At this stage, any required targeting ligands, imaging modalities or therapeutic agents can be anchored to its surface in order to achieve the desired function (Veiseh et al., 2010). In order

to create SPIONs that are capable of targeting and or eliminating tissue (if it is damaged) very detailed attention needs to paid to their design parameters such as size, shape, coating and surface modifications (Dobrovolskaia et al., 2008; Chouly et al., 1996). Only with such careful engineering can you create nanoparticles that are able to overcome various biological barriers and carry out their functions. The drug delivery systems require additional consideration given to interactions between nanoparticles and target cells and the controlled release of the drugs (Veiseh et al., 2010).

Design Considerations: Before researchers can begin the synthesis of MNPs, they must understand the nature of nanoparticles as pharmaceutical or therapeutic constructs that must travel through the body to find their target, can overcome various biological barriers in the process and their design parameters in order to develop an effective and efficient MNP system.

In Vivo Barriers: Human body's defense system has created a number of so called biological barriers in order to protect itself from foreign entities entering the body, which coincidently include injected therapeutics or any other agents, and very successfully prevents them from reaching their target destinations (Ferrari, 2005). These biological barriers have the ability to significantly hinder nanoparticle function up to a point where they can be rendered non-functional and be secreted out of the body. The body's defense system does so by inhibiting nanoparticle movement, causing physical changes to them or by inducing a negative host response using a number of biochemical signaling pathways (Belting et al., 2005). Nanoparticles first come into contact with blood once they are injected. Here, the heterogeneity of blood can cause nanoparticles to agglomerate resulting in alterations to their magnetic properties ultimately leading to their sequestration. There is also a possibility of nanoparticles interacting with plasma proteins or various other non-targeted cell surfaces (Davis et al., 2002). Each one of these

barriers poses a threat of non-targeted premature binding and possible endocytosis of nanoparticles before they can reach their target.

These barriers can also present themselves in terms of various anatomical size restrictions which the nanoparticles must comply with in order to reach their target cells (Ferrari et al., 2005). One particular example refers to the interaction of nanoparticles with the blood brain barrier (BBB). Besides posing very strict physiochemical restrictions on the nanoparticles, BBB only allows nanoparticles with a sufficiently smaller size to pass through (Koo et al., 2006). A lot of nanoparticles have been engineered that can overcome these extracellular barriers and successfully bind to the surface of their target cell which in many cases initiate receptor-mediated endocytosis. Once inside the cell, they must now face intracellular barriers as well. A number of physiological pathways here can lead to acidification of endosomes resulting in destruction of the nanoparticles that are carried inside (Bareford et al., 2007). Nanoparticles can also be translocated from endosomes into lysosomes where hydrolytic and enzymatic reactions can completed metabolize them. As long as researchers are mindful of these biological barriers and see them as demands put upon the engineering of nanoparticle core and surface properties, they can overcome all of these limitations (Veiseh et al., 2010).

Physiochemical Considerations: Nanoparticle's *in vivo* functionality is largely dependent upon its physiochemical properties which include its morphology, size, charge and various other surface properties. All of these properties must be considered at each stage of MNP design and how they will contribute to its overall physiochemical properties (Veiseh et al., 2010). For instance the shape of MNPs can have significant effects on their bio-distribution and even their bioavailability. One research study suggests that anisotropically shaped MNPs are better at avoiding bio-elimination compared to their spherical counterparts (Liu et al., 2006) which

subsequently results in longer bioavailability. Another study showed that increased length-to-width aspect ratio of MNPs results in increased blood circulation time (Geng et al., 2007). Variations in hydrodynamic size of MNPs can have similarly significant effects on their bioavailability and distribution as well. Research has shown that hydrodynamic size of nanoparticles helps in governing their concentration profile in blood vessels (Decuzzi et al., 2005; Decuzzi and Ferrari, 2006; Decuzzi and Causa, 2006), plays a significant role in their clearance from circulation (Long mire et al., 2008; Choi et al., 2007; Moghimi, 1995; Mogimi, Hunter et al., 2001; Zamboni., 2008; Moghimi, Moein et al., 2001) and most obviously dictates the permeability of nanoparticles (Chavanpatil et al., 2006).

Other surface properties of nanoparticles such as charge and hydrophobicity or hydrophilicity can also have major effects on their interaction with the in vivo environment. These properties can either limit or enhance nanoparticle interactions with the immune system, plasma proteins, extracellular matrices and non-targeted cells (Davis, 2002). Various studies have shown that charged and/or hydrophobic nanoparticles have shorter circulation times due to enhancement of these interactions (Chouly et al., 1996). Positively charged nanoparticles are also at the risk of binding with non-targeted negatively charged cells which can prevent them from reaching their target site (Veiseh et al., 2010). However, research over the past few years has led to the development of stealth nanoparticles whose surfaces are modified with molecules like polyethylene glycol (PEG) resulting in prolonged circulation times (Harris et al., 2003). Some other organic surface coating molecules include dextran, chitosan, liposomes, micelles and copolymers. All of these molecules function to serve a similar purpose (Veiseh et al., 2010).

Specifically, variations of PEG have been used in a number of FDA approved pharmaceutical formulations (Fuertges et al., 1990). These variations are neutral and hydrophilic and function to

improve the dispersity and bioavailability of MNPs based on the principles mentioned above. MNPs that have been coated with PEG are commonly referred to as stealth nanoparticles because of their increased ability to evade detection by the reticuloendothelial system (RES) (Harris et al., 2003). Although this limits their usage in imaging RES related cells (Papisov et al., 1993), it makes them perfectly suitable for use in target-specific cell labeling following modification with targeting ligands (Sun, Fang et al 2008; Sun, Veiseh et al., 2008; Chen et al., 2007). MNPs used in this research have been coated with a variation of PEG called polyethylene glycol diacrylate (PEGDA). The design process follows from a polymeric design strategy that considers both: efficient surface coating and functionalization (Veiseh et al., 2010). This idea was first researched by Kohler in early 2000s (Kohler et al., 2004). He was able to prepare a variation of PEG that could be attached to SPION surface via covalent bonding at one end and the other end could be functionalized by any of the various therapeutic agents or targeting ligands (Sun, Fang et al 2008; Sun, Veiseh et al., 2008; Sun et al., 2006; Veiseh et al., 2005; Gunn et al., 2008; Veiseh et al., 2008).

Directing nanoparticles in vivo: Variations of passive, active and magnetic targeting have been used to direct the travel of nanoparticles inside the body (Veiseh et al., 2010). First of the three, passive targeting, does not require an input of energy, instead it makes the use of a certain nanoparticle's predetermined physiochemical properties to guide its path to the target cell or tissue. Cancer research involving nanotechnology has seen use of this passive approach. Solid tumor tissue can be targeted relatively easily by passive targeting due to its certain properties. In most cases the goal of tumor cells is to grow rapidly which requires the production of new blood vessels. To achieve this rapid growth, the neovasculature that is produced quickly is usually poorly organized and contains leaky fenestrations. Along with other small macromolecules,

nanoparticles can use these fenestrations to enter the tumor cells (Jain, 1999 and 2001). Another effect of this hurried growth is the development of an inefficient lymphatic drainage, which results in lower clearance of the nanoparticles that have entered this vasculature resulting in their accumulation at these sites (Hobbs et al., 1998; Mcneil, 2005). This overall phenomenon is known as enhanced permeation and retention (EPR) (Maeda et al., 2000). However, the successful application of EPR is dependent upon a number of things including degree of fenestration and lymphatic drainage rate and its use is limited to specific solid tumors (Veiseh et al., 2010).

Due to the limitations of passive targeting further modification of nanoparticles was pursued. As mentioned above, surfaces of nanoparticles can be modified with molecular ligands such as PEG; additional modification can also be done using specific molecular targeting ligands. This overall approach represents, second of the three approaches mentioned above, active cell targeting (Zhang et al., 2002; Sinha, 2006). These targeting ligands are complementary to unique receptors on surfaces of the target cells and therefore, actively target only the diseased cells. Successful application of the active cell targeting depends upon the type of ligand used and its density and by the size and shape of the nanoparticles. These factors become even more important because of their role in the multivalency phenomenon. Multivalency refers to the enhanced binding avidity phenomenon observed when multiple targeting ligands bind to multiple receptors between two surfaces at the same time (Wright et al., 2001; Munson et al., 1979). A research study done in mid 2000s showed the role that the multivalency phenomenon plays in nanoparticle binding. It indicated that the density and molecular organization of the targeting ligands on nanoparticle's surface has a major role in its binding to the target cell (Hong et al., 2007). Simultaneous ligand binding increases with increasing the density of the targeting ligand, but only up to a certain

limit, after that multivalent interactions could be sterically hindered (Montet et al., 2006). Figure 2-7 shows how nanoparticle size and shape along with ligand density might affect the multivalency. Illustrations depicted in Figure 2-7 are based on the work of Jiang et al (Jiang et al., 2008). This study looked at nanoparticles ranging in size from 2 nm to 100 nm. Nanoparticles under the size limit of 25 nm do not possess the ability to be multivalent as their size permits them from having multiple ligands on their surface. Conversely larger nanoparticles do possess the ability to be multivalent, but they face difficulty in the endocytosis process which limits their functionality for certain applications. Nanoparticles of 25 – 50 nm size were shown to be most efficient as they can show multivalency binding effects and can be endocytosed relatively easily. Our research group used nanoparticles of two different sizes, 200 nm and 500 nm for comparison. As insulin receptors are found on the cell surface our research group was more concerned with their ability to be multivalent rather than any issues associated with endocytosis. In fact, for the bigger picture concept we would prefer to have nanoparticles that cannot be endocytosed as it will increase their bioavailability. Insulin receptors are found on the surface of the cells. Therefore, there is not a need for them to be endocytosed. A bigger size of nanoparticles prevents them from endocytosis and might even provide even longer bioavailability.

Finally, researchers have also used external magnetic systems to guide MNP localization in an approach called magnetic targeting (Dobson, 2006; McBain et al., 2008). Powerful magnets are used to create a magnetic field which can result in accumulation of MNPs within that area. One limitation of this approach is that it becomes less and less effective as you go deep into the body due to the loss of magnetic field strength. Studies that have seen optimal use of this technology focus on target sites that are closer to the skin because of the higher strength of the magnetic

field as the area is closer to the magnetic source. 2004 saw successful implementation of this approach in a clinical trial aimed at delivery doxorubicin, a chemotherapeutic agent, to hepatocarcinoma cells (Wilson et al., 2004).

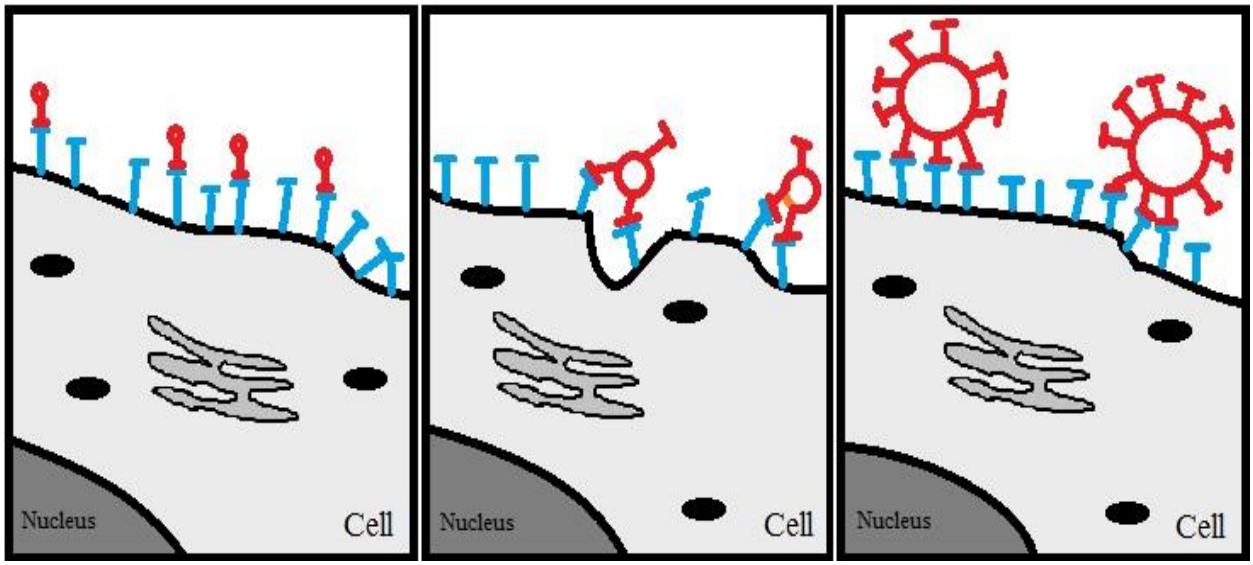

Figure 2-7: Illustration of the multivalent capabilities of different sized MNPs. Illustration on the left represents nanoparticles of ~ 25nm, in the middle represents nanoparticles of 26 – 70 nm and on the right represents nanoparticles of 71+ nm and their multivalent abilities.

Other Considerations: As mentioned before nanoparticles used for drug delivery require some additional considerations given to drug loading and release. First of all, MNPs created for drug delivery must have the capability to carry and protect the drug payload. The type of MNP coating and loading method (e.g. covalent bonding, linker/click chemistry or physical interactions such as any affinity interactions) determine the MNP's ability to carry and protect the payload (Veiseh et al., 2010). Secondly, the MNPs carrying drugs must be able to overcome the cellular drug resistance. Multiple drugs can be loaded onto a single MNP to help in overcoming this resistance. However, loading multiple drugs requires planning to accommodate the different therapeutic agents (Veiseh et al., 2010). Finally, the drug release mechanism must

be optimized in order to achieve maximal efficacy. Different approaches have been used to optimize the drug release ranging from predetermined drug release to the use of a feedback loop (drugs released in response to certain changes in the cellular environment). Figure 2-8 below shows the basic structure of a multifunctional imaging/therapeutic MNP and the local activity of different categories of therapeutic agents. MNPs designed for such dual applications may carry targeted moieties attached to polymeric tethers (e.g. PEG), multiple imaging agents (optical, radio or magnetic) and other bio- or chemotherapeutic agents. Once in vivo; different mechanisms can be activated dependent upon the choice of therapeutic agents used.

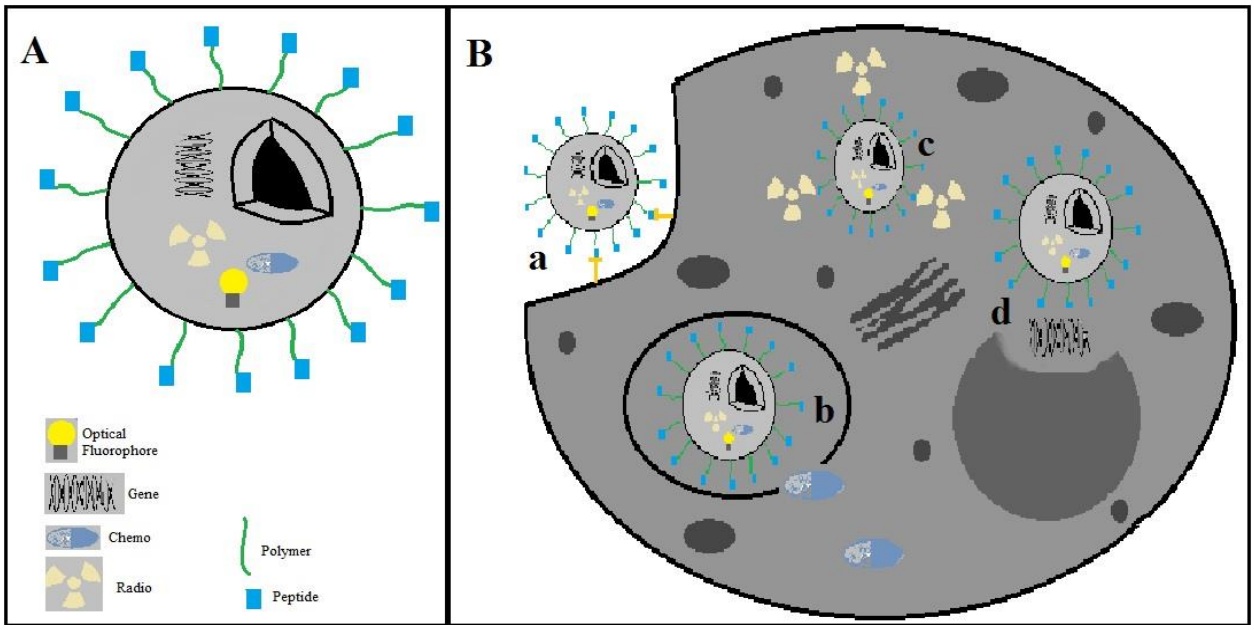

Figure 2-8: Illustration of the basic structure of a multifunctional MNP and the local activity of different therapeutic agents. (A) Basic Structure, (B) Local intracellular activity, (a) MNP binding to surface receptors, (b) controlled release of chemotherapeutics, (c) decay of radioactive materials, (d) controlled release of gene therapeutic materials.

One of the final things that needs to be considered in MNP design is the matter of toxicity to the body. In order to make sure that a nanoparticle system does not pose any threat to the human

body, toxicity of all of the individual components and the nanoparticle as a whole must be evaluated. This is necessary because we need to consider how a nanoparticle will interact with the body during its bioavailable phase and also after its degradation starts and individual components start getting excreted (Lewinski et al., 2008).

Surface Modification: Various chemical approaches have been employed to achieve the tethering of imaging, targeting or therapeutic agents onto nanoparticles. Most of these approaches can be divided into two categories: covalent linkage strategies and physical interactions. The approach used depends mainly on the chemical properties and functional groups present on the coating polymers and the ligand to be attached. The primary goal of the surface modification chemistry is to attach the agent without compromising its functionality which is, in part, determined by the manner in which the ligand is attached to the nanoparticle. For example, in case of antibodies, if they are attached in such a way that their recognition site is hindered or shielded, they will lose their ability to bind to the antigen (Veiseh et al., 2010).

Covalent linkages are strong and stable bonds that are formed between functional groups on the nanoparticle surface and the conjugated ligands. Usually the functional groups, typically amino, carboxylic acid and thiol groups, are added to the nanoparticle surface through its polymer coating. The type of polymer coating used determines the type and number of functional groups present on the surface of nanoparticles. These functional groups can either be part of the body of the polymer (e.g. chitosan) or they can be found at their terminal ends (e.g. PEG). These functional groups are also present on the imaging, targeting or therapeutic moiety to be attached. There are three methods available to create a covalent linkage, and first one is called direct nanoparticle conjugation. In this approach, the functional groups on the nanoparticle surface are either directly bonded to the moieties or a linkage reaction is facilitated with the help of a

catalyst. Although nanoparticle surfaces functionalized with amine, sulfhydryl, aldehyde and active hydrogen groups can be targeted, except for amine-functionalized nanoparticles, they are at risk of intercalation or cross linking.

Click chemistry is the second method that can be used to achieve this conjugation. This technique was developed to make conjugation reactions between bioactive surfaces easier and less harsh to biomolecule ligands as they require mild reaction conditions and create biocompatible linkages (Hein et al., 2008). This method provides some unique advantages including the high specificity of azide and alkyne reactive groups for each other and uncreativeness towards most other functional groups. The bonds created using click chemistry are highly stable and extremely rigid which prevents cross interactions by helping to maintain the conformation of attached agents (Von Maltzahn et al., 2008; Sun et al., 2006). This method presents the ideal choice for applications where orientation and stability of the attached moiety are significantly important.

Linker chemistry is the third method of creating covalent linkages. Its unique advantage lies in the control that it provides over the molecular orientation of bound agents which is a significant aspect of protecting targeting ligand functionality. The most common approach in linker chemistry involves a linker molecule that binds the amine group on the surface of a SPION with the sulfhydryl group of the binding agent or the cystine amino acid is targeted for reaction when the binding agent is a peptide or a protein. Linker chemistry is ideal for use in cases where a reaction with a complex biological molecule is required. This prevents over-labeling and reactions with multiple reactive sites (Veiseh et al., 2010).

In contrast to covalent linkages, physical interactions include any electrostatic, hydrophilic/hydrophobic and affinity interactions. Physical interactions are accompanied by rapid speed of binding, high efficiencies and do not require any intermediary modification steps.

Attachment of insulin onto MNPs

The idea of insulin's attachment onto the MNPs was explored further by Gupta et al (2003). This research group worked to couple insulin with superparamagnetic iron oxide nanoparticles of certain size and shape for targeting surface receptors on cells and thereby preventing endocytosis of the NPs. 1-Ethyl-3-(dimethylaminopropyl)-carbodiimide (EDCI) coupling method was used to covalently attach insulin onto the NP surface. This coupling method results in a fairly tight connection between insulin and the nanoparticles. This tight connection may affect the activity or functionality or conformation of the insulin molecule, as the tight connection can likely intervene the insulin. To reduce the likelihood of intervening the insulin from the nanoparticles, a soft chain made up of PEGDA was added between the MNP and the insulin molecule, which differs from the work of Gupta et al (2003).

In sum, Section 2.5 provided a detailed analysis of the two potential capturing techniques, immunoprecipitation and the use of MNPs, and concluded that the latter approach was taken in this thesis study, thereby achieving part of Objective 1 of the thesis (see Chapter 1).

Chapter 3: Materials and Methods

3.1 Materials

3.1.1 Insulin

Manufacturer Name:	Sigma-Aldrich
Name in Manufacturer's Catalogue:	I5500 – Insulin from bovine pancreas - ≥25 USP units/mg (HPLC), powder
Empirical Formula:	$C_{254}H_{377}N_{65}O_{75}S_6$
Formula (Molecular) Weight:	5733.49 g/mol
Properties:	White, Powder form, Zinc cation traces of ≤1.0%, Storage temperature of -20^{o}C.

3.1.2 NH_2 functionalized magnetic nanoparticles

200 nm and 500 nm NH_2 functionalized magnetic nanoparticles reacted with PEGDA (NH_2-MNP-PEGDA) were available to me through our research team member, Ruixue Yin. The following procedure describes her methodology (Yin et al., 2014):

a. NH_2 functionalized magnetic nanoparticles were obtained from Aladdin Reagent Co. Ltd Shanghai, China.
b. 0.5 g PEGDA was added to 5 mL PBS (pH 7.4).
c. 1 mL NH2 functionalized magnetic nanoparticles were added to the mixture.
d. Solution was stirred at room temperature for 24 hours.
e. After the reaction was over, the nanoparticles were concentrated by a magnet and washed for three times.

If insulin was attached directly onto the magnetic nanoparticles, there might have been a possibility of MNPs hindering insulin's structure or activity due to their close proximity. Addition of the PEGDA soft chain results in insulin's attachment further away from the MNPs and prevents insulin from facing any major effects of MNP's physiochemical properties.

3.1.3 General Laboratory Chemicals Used

- Hydrochloric acid (HCl) 37.5 % concentration
    - HCl was used to match the isoelectric point of the solution to that of the insulin used. Without using HCl, insulin would have precipitated in the PBS solution.
- Phosphate buffered saline (PBS).

3.1.4 Dialysis sacks

Manufacturer Name:	Sigma-Aldrich
Name in Manufacturer's Catalogue:	D6191 – Dialysis sacks
Pore size:	12000 Da MWCO
Properties:	Average flat width 25 mm, inflated diameter approximately 16 mm; pre-cut, open ended; dry; unwashed; approximate length of 30 cm (12 in.); will retain proteins with M.W. > 12000 Da.

Dialysis was chosen as a method to get rid of the unbound insulin from the solution. Dialysis sacks combined with the tubing closures provided a gentle method to remove the unbound insulin. If the unbound insulin was left in the solution it would have resulted in convoluted results.

3.1.5 Dialysis tubing closures

Manufacturer Name:	Sigma-Aldrich
Name in Manufacturer's Catalogue:	Z371092 – Dialysis tubing closures
Properties:	Blue; 50 mm; operating temperature range of 0 – 90^{o}C; made of Nylon 66.

These tubing enclosures were a part of the dialysis kit.

3.1.6 General laboratory equipment used

Beakers, magnetic stirrers, magnetic stirring rods, weighing scale, pipettes, and plastic tubes. All of this equipment was used to successfully complete the experiment.

3.2 Procedure for Synthesis

Step 1: 1 mL 0.01 M HCl was added to 5 mg Insulin.

Step 2: 4 mL PBS was added to the solution.

Step 3: 1 mL 200 nm NH_2-MNP-PEGDA were added to the solution. Note that NH_2-MNP-PEGDA underwent ultrasonic treatment for 5 minutes before addition to the solution.

Step 4: The solution was stirred at room temperature for 24 hours.

Step 5: The resulting solution was put into a dialysis tube for 24 hours to get rid of the unbound insulin.

3.2.1 Parallel Experiments

Parallel experiments were run with 500 nm NH_2-MNP-PEGDA. The only change to the synthesis procedure here was the replacement of the 200 nm NH_2-MNP-PEGDA with 500 nm NH_2-MNP-PEGDA. Each of these experiments was repeated five times and results were observed to be very similar. For simplicity purposes, the results from one experiment of each kind are presented below.

Parallel experiments were also run in the absence of any nanoparticles at all. These experiments just involved insulin going through the synthesis procedure; the steps involving NH_2-MNP-PEGDA were taken out. The purpose of this parallel experiment was to gather information about insulin, so it can be compared to information gathered regarding the insulin – NH_2-MNP-PEGDA complex.

Five different experiments were run in total and hence five different sets of spectra were generated and analyzed. Due to clarity, simplicity and conventional purposes, only spectra from the best sample set are presented below. A simple version of the methodology can be seen below in Figure 3-1.

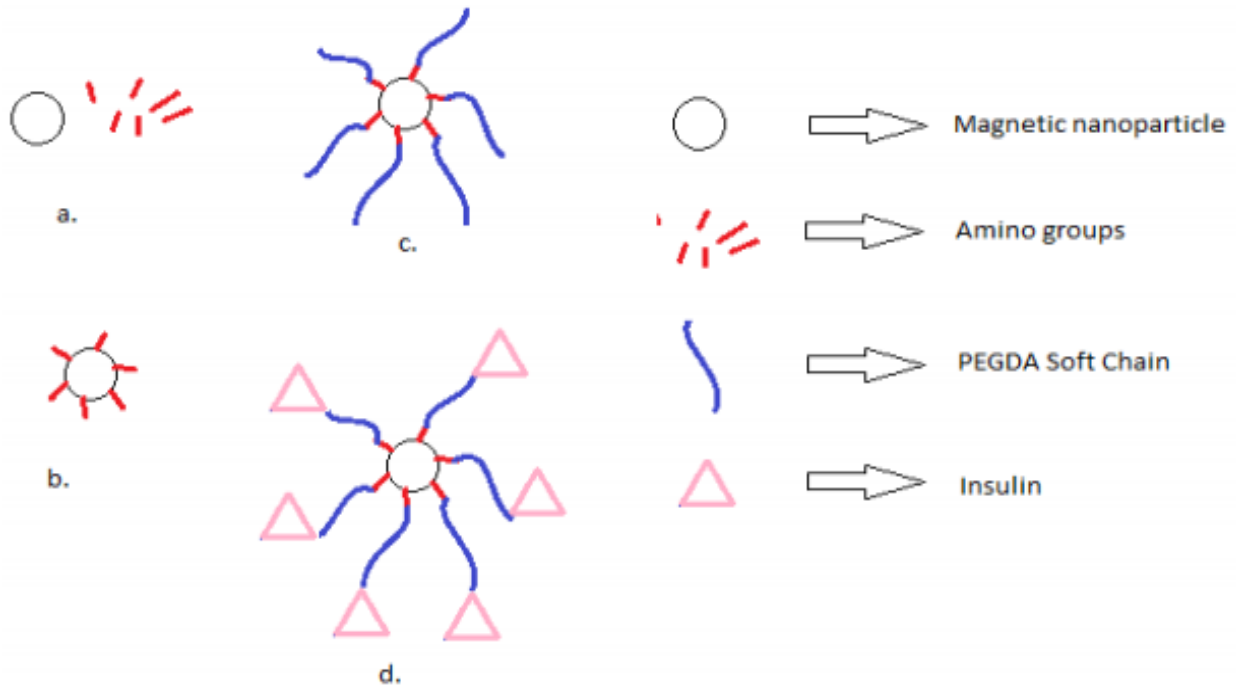

Figure 3-1: Representation of the MNP-Insulin complex synthesis. (a) magnetic nanoparticles and amino groups, (b) NH_2 functionalized magnetic nanoparticles, (c) NH_2 functionalized magnetic nanoparticles with PEGDA soft chain added, (d) final product: NH_2 functionalized

3.3 Characterization

3.3.1 Fluorescence spectrum

Fluorescence measurements were carried out on a Pistar-180 CD spectrometer (Applied Photophysics Ltd., Leatherhead, UK) at room temperature using a 0.100 cm optical path length cuvette (Hellma, 106-QS). Protein solutions were scanned from 290 – 270 nm region in 1 nm steps. The baseline was measured using 1X PBS.

Fluorescence spectrum was used to confirm the presence of insulin in our sample solutions. If the insulin-MNP attachment had failed, dialysis would have removed all of the insulin from the

solution. Fluorescence spectroscopy was used to confirm that insulin was present in the solution, which refers to *Objective 1* of this thesis. Fluorescence spectroscopy provides a fast and simple method to measure the concentration of a particular substance in a sample solution based on its fluorescent properties. Fluorescence spectrometry can be used for a number of applications, which might require a range of analyses; either very complex or very simple. A complex analysis might involve using the fluorescence spectrum of an unknown compound and using the data to try to identify certain characteristics in it, which will point towards the identity of that compound. Fluorescence absorption spectrum helps us determine the identity of the unknown compound by giving information regarding which wavelengths were absorbed by the sample. Different compounds absorb different wavelengths, which gives them a unique identity and can be used to identify them. Fluorescence emission spectrum could also be used for the same purpose. However, the emission spectrum provides information, regarding which wavelengths are emitted by the unknown compound after absorbing the initial light. The wavelengths emitted are once again specific for certain compounds and can be used to identify them. An example of a relatively simple analysis is where the goal is to just determine the concentration of a compound that is already known to be present in the sample solution (Hooijschuur, n.d).

3.3.2 Absorbance spectrum

Absorbance measurements were carried out on a Pistar-180 CD spectrometer (Applied Photophysics Ltd., Leatherhead, UK) at room temperature using a 0.050 cm optical path length cuvette (Hellma, 106-QS). Protein solutions were scanned from 260 – 190 nm region in 1 nm steps. The baseline was measured using 1X PBS.

Absorbance spectroscopy is an analytical tool employed by chemists to measure the concentration of a certain substance in a sample solution, which can be used further to quantify the amount of the substance present. The absorbance spectrum shows how much light of a particular wavelength is absorbed by the sample solution. Although the data presented below can be used for the purpose of finding the exact concentration of insulin that was present in the solution by using the Beer-Lambert Law, this research did not have a need for that. Before moving onto more time consuming spectroscopies, we wanted to confirm the presence of insulin in our solution.

Absorbance spectrum served a similar function to the fluorescence spectrum. Results from both spectroscopies were used to confirm the attachment of insulin onto the MNPs. The only reason an absorbance or fluorescence spectrum would indicate the presence of insulin in the solution is only if it was attached onto the MNPs otherwise it would have left the solution during dialysis, which refers to our *Objective 1*.

3.3.3 CD spectrum

CD measurements were carried out on a Pistar-180 CD spectrometer (Applied Photophysics Ltd., Leatherhead, UK) at room temperature using a 0.050 cm optical path length cuvette (Hellma, 106-QS). Protein solutions were scanned from 260-190 nm region in 0.5 nm steps at a scan rate of 5 nm min^{-1} and a bandwidth of 6 nm. The baseline was measured using 1X PBS. CD spectra displayed are an average of 4 spectra, and given in molar ellipticity (Θ) units.

CD spectra are displayed as molar ellipticity ([θ]), which has units of deg cm^2 $dmol^{-1}$, and is calculated by the following equation:

$$[\theta] = \frac{100\,\theta_{obs}\,MW}{c\,b} \qquad \text{(Equation 3.1)}$$

where θ_{obs} is the measured ellipticity (deg), MW is the molecular weight of the protein (g mol^{-1}), c is the protein solution concentration (mg mL^{-1}), b is the cuvette's pathlength (cm). The value of 100 originates from the conversion of molar concentration to dmol cm^{-3} concentration.

Circular dichroism spectroscopy (CD) has a wide range of applications, however it is mainly used to investigate the secondary structure of proteins. It can very quickly provide information regarding the folding properties of proteins due to its ability to detect and quantify the proportion of alpha helices to beta sheets to random conformations. Protein CD is most widely used to determine whether or not the expressed protein, insulin in our case, is in its native conformation or if any mutations have occurred. This capability of CD is the reason that it is frequently used in the biopharmaceutical industry as a characterization tool. CD spectrums are used to study the effects of manufacturing processes, formulation compositions and conditions, storage conditions and delivery systems on protein conformation (Li et al., 2011). Different structural elements such as α-helices, β-sheets, etc., of proteins have characteristic CD spectra, which means if a protein structure is dominated by either one of the structural elements, its CD spectrum will be characteristic of that element. For instance, α-helical proteins display negative bands at a wavelength of 222 nm and 208 nm with a positive band at 195 nm. Proteins dominated by β-sheets display negative bands at 218 nm and positive bands at 195 nm. Finally, proteins that are dominated by random structural elements display very low ellipticity above 210 nm (Greenfield, 2007).

If a certain protein was to undergo any mutation or conformational change, it would result in a shift in the placement of its positive or negative bands. For instance, if a protein whose structure is predominantly defined by β-sheets undergoes such conformational changes, its negative bands might not be found at 218 nm and its positive band might not be present at 195 nm, which as

mentioned above is the characteristic of proteins whose structure is dominated by β-sheets. As a CD spectrum of a protein provides information regarding its structure, especially the secondary structure, it was used to check if the insulin's conformation had undergone any changes during the synthesis processes. This would provide information regarding *Objective 2* of this thesis.

3.3.4 FTIR spectrum

FTIR measurements were performed on a Biorad FTS-40 spectrometer using diffuse reflectance infrared Fourier transform (DRIFT) mode. Samples were mixed with spectroscopic grade KBr in an approximate 1:10 ratio. FTIR spectra were an average of 256 spectra collected at room temperature in the 4000-400 cm^{-1} range using 4 cm^{-1} resolution.

FTIR spectrum was also used because it would provide information regarding *Objective 2*. FTIR spectrums are used to study any conformational changes in protein structures. Fourier-transform infrared spectroscopy (FTIR) is used in a number of applications. Initially it was used for the analysis of organic compounds in order to gain information regarding their molecular structure, chemical bonding and molecular environment (Gerwert et al., 2010). FTIR's applications today range from its use in analysis of proteins, small molecules or molecular complexes all the way to use in analysis of cells or tissues. The analysis of proteins provides information regarding the protein conformation, folding and even the molecular details from protein active sites during enzyme reactions (Siebert et al., 2008). The data obtained through FTIR can be used to study conformational changes in proteins, hydrogen bonding interactions of cofactors and redox intermediates, proton transfer coupled to electron transfer, identification and properties of metal ligands, and even the role that water molecules play (Berthomier et al., 2009).

Although FTIR spectrum provides information regarding α-helices, it is particularly sensitive to the β-structures and any changes or mutations that they might present within a protein's structure. Therefore, it pairs well with circular dichroism, which as mentioned above is more sensitive towards α-helices. FTIR and CD spectroscopy are highly complementary towards each other, which is why both of them are used fairly commonly to study protein structures (Gerwert et al., 2010).

Chapter 4: Results and Discussion

4.1 Fluorescence Spectroscopy

Our purpose of using fluorescence spectroscopy was relatively simple. As presented in methodology, dialysis was used to get rid of the unbound insulin and if the attachment was not successful it would have gotten rid of all of the insulin. The fluorescence absorption spectrum was used to confirm that there was insulin present in our sample solution. The only reason any insulin would be left in the solution is if it was attached onto the MNPs. Therefore, the results in Figure 4-1 below also confirm the attachment of insulin onto the MNPs. Achievement of these

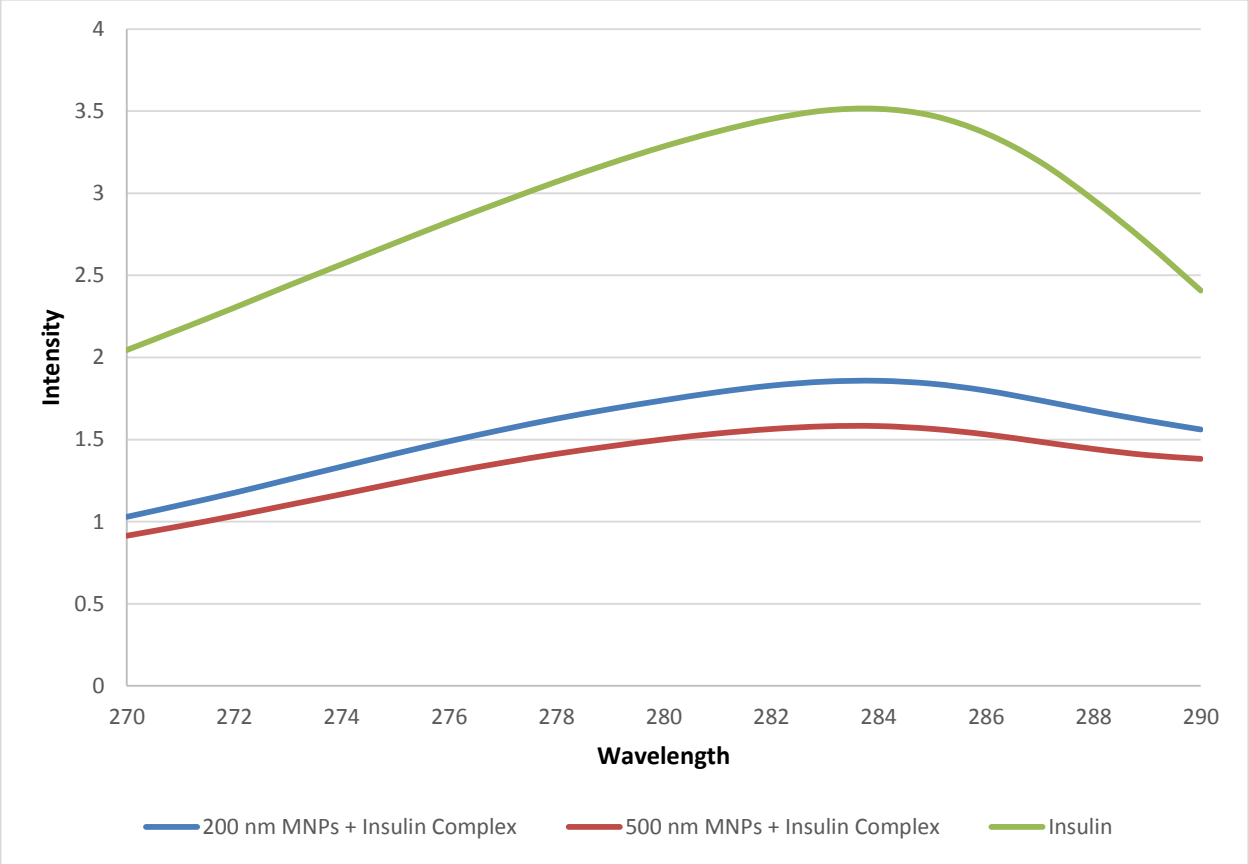

Figure 4-1: Fluorescence spectra of insulin, 200 nm MNP + Insulin Complex and 500 nm MNP + Insulin Complex.

results refers to Objective 1.

The reason that the insulin curve is much higher compared with the insulin-nanoparticle complexes is because insulin was at a much higher concentration compared to other samples. Insulin was at a higher concentration because the sample with just insulin did not go through a dialysis tube. There was not a feasible method present for us to calculate the concentration of insulin after dialysis. Therefore, I left the insulin concentration at the level it was before performing the dialysis. For the purposes of my study it was not necessary to have an exact concentration of insulin. One possible option to try and gauge the right amount of insulin was to dilute the insulin sample in a serial manner and once again run the different characterizations to see which one was the closest. Although I ran the serial dilutions I was not able to obtain a fluorescence spectrum for it due to the issues regarding laboratory equipment. However, the results in Figure 4-1 provide us with enough information regarding our questions. Insulin curve is higher and that is what we expected compared to the other two curves. A little difference between the 200 nm and 500 nm samples can also be attributed to slightly different concentrations of insulin being present in the solution. Despite the differences in the curves, we can safely say that insulin was present in each of our sample solutions and was ready to move onto next tests.

4.2 Absorbance Spectroscopy

The absorbance spectrum gives information regarding the signals given off by the aromatic rings in a certain protein. Different proteins absorb light at different wavelengths, which is why they project distinct absorbance spectra. The curves in Figure 4-2 show that there was a substance present in all three of the solutions and because the curves follow the same trajectory and have their peaks around the same wavelength it is safe to assume that all three contained the same

substance, insulin. The main reason for obtaining the absorbance spectra was to confirm the presence of insulin in our samples, which also confirms the attachment of insulin onto the MNPs. A dialysis procedure was used to get rid of any unbound insulin from the sample solutions. If the attachment of insulin and MNPs had not been successful, all of the insulin would have escaped the dialysis sack because insulin has a much smaller size compared to the pores in the dialysis sacks. The absorbance spectra would not have shown the presence of any substance. The dialysis sacks used for the experiment had a pore size of 12000 Da, which means they would have retained any proteins bigger in size than 12000 Da and any smaller proteins would have escaped the sack. Insulin used had a molecular weight of 5733.49 Da and therefore would have escaped the dialysis sacks unless it was bound to something bigger. According to the curves in Figure 4-2

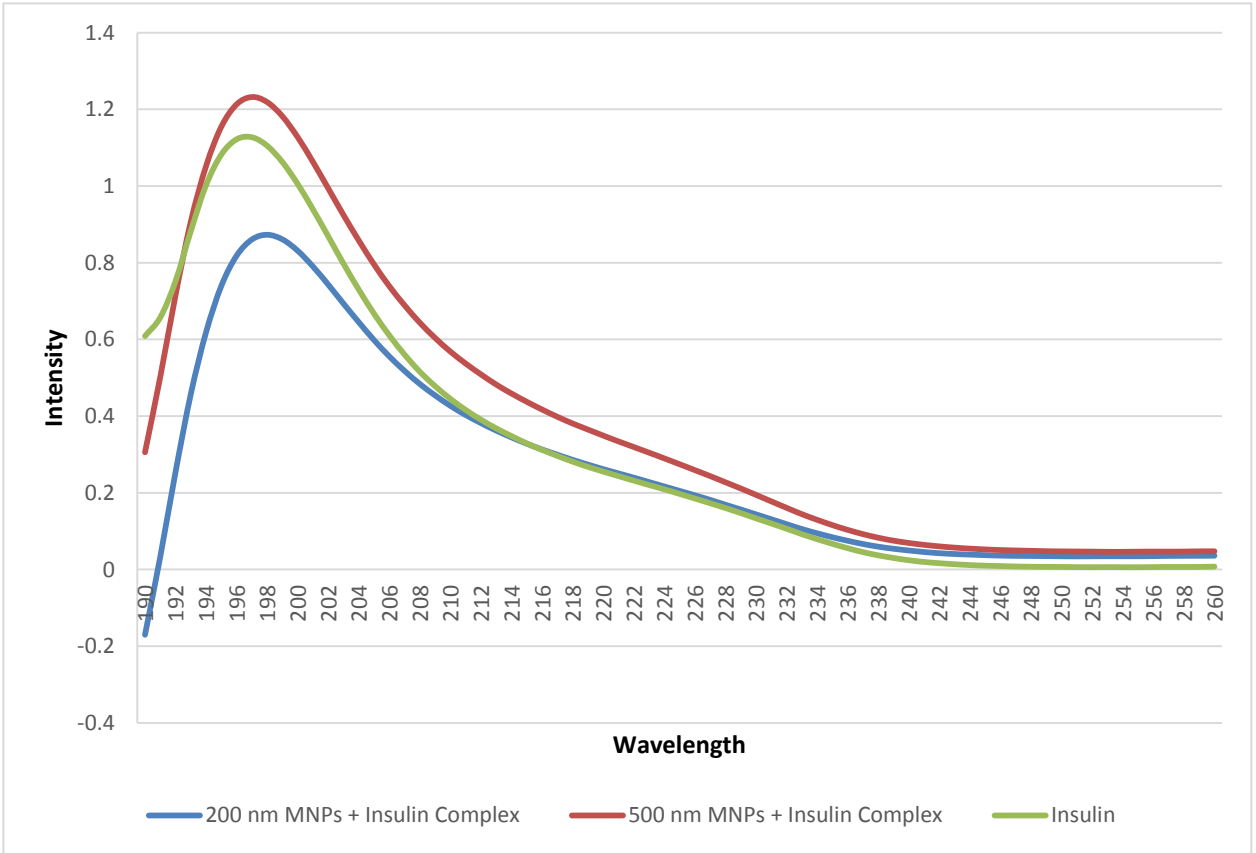

Figure 4-2: Absorbance spectra of insulin, 200 nm MNP + Insulin Complex and 500 nm MNP + Insulin Complex.

insulin was present in all three samples. The only reason that insulin would have been able to stay in the solution is if it had attached onto the MNPs, therefore the absorbance spectra below also confirm the attachment of insulin onto the MNPs. Achievement of these results further confirms the results of the fluorescence spectrum and refers to Objective 1.

4.3 Circular Dichroism Spectroscopy

Insulin has an alpha-helical secondary structure which means that it should have distinctively positive bands at 222 nm and 208 nm and a negative band near 195 nm. The results presented in Figure 4-3 show that the curves follow these parameters. All three curves presented below have their valleys very close to 222 nm and 208 nm, while the peak is towards 195 nm. Just as with the fluorescence spectrum results, these results could be analyzed in a much greater detail and a complex manner, however the purpose of this research was to confirm that no conformational changes took place after the attachment of insulin onto the MNPs. The fact that all of the curves have their peaks and valleys very close to each other means that no conformational changes took place in insulin's structure due to its attachment onto MNPs. Any shifts between these peaks and valleys would have indicated a conformational change in insulin.

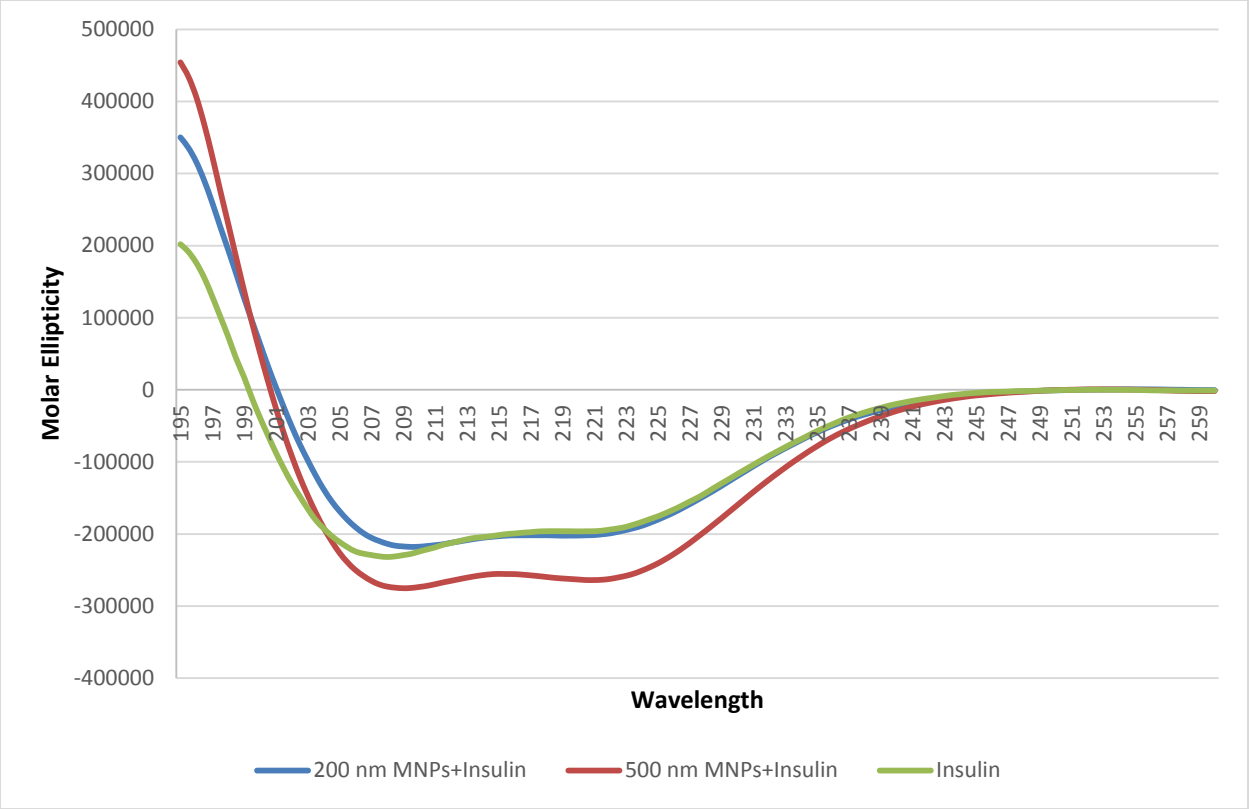

Figure 4-3: CD spectra of insulin, 200 nm MNP + Insulin Complex and 500 nm MNP + Insulin Complex.

Furthermore, the CD data obtained from experiments was analyzed using CDNN – Version 2.0, a specialized software designed to analyze data from CD spectra. Output from the software shows the CD spectrum and an estimate of the relative content of its secondary structural elements. Although the output presents estimates for different subsets of the full wavelength range, we opted to use the full wavelength range, 195 – 260 nm as it would provide the most comprehensive analysis. Also, the lowest wavelength prediction is proposed to be most reliable (jenalib.leibniz-fli.de, n.d). Table 1 below presents the output received from CDNN, net using 33 basespectra (complex CD spectra). Ideally, the total sum of the secondary structural elements should be as close to 100% as possible. However, there is some room for error made by the program. As long as the total sum values presented do not deviate more than 5 – 10% from

100%, the data can be considered reliable. As Table 1 shows all three samples run for this research are well within the 5 – 10% error margin, the data presented below can be considered reliable. Moreover, the percentage represented for each structural element should also ideally be the same for all of the samples. However, just as with the total sum values the program allows some error margin, specifically if the data was obtained with in the 195 – 200 nm wavelength range and if the complex CD spectra were used, the error margin is 6.20%. More detailed error margins for different ranges and other spectra are presented in the appendix. The data in Table 1 shows that percentage of each structural element for all three samples is within the 6.20% error margin. When the 200/500 nm MNPs + Insulin complexes are compared with Insulin samples according to the data below it can be safely said that insulin did not undergo any conformational changes as each structural element is still present within its percentage range when considering the error margin. Achievement of these results refers to Objective 2.

Table 4-1: Secondary structural element percentages.

Complex	**Insulin**	**200 nm MNPs + Insulin**	**500 nm MNPs + Insulin**
	195-260 nm	195-260 nm	195-260 nm
Helix	11.60%	11.50%	13.20%
Antiparallel	31.30%	34.00%	30.90%
Parallel	5.50%	5.80%	5.90%
Beta-Turn	19.70%	18.70%	18.90%
Random Coil	33.50%	32.50%	32.50%
Total Sum	101.70%	102.60%	101.50%

4.4 Fourier-transform Infrared Spectroscopy

The peaks and valleys of the curves below represent data points that can be used for very complex analytical purposes. For instance the wavenumber (cm^{-1}) range presented on the x-axis can be broken down into various regions which can be looked into with further detail to identify alpha-helices or beta-sheets and even postulate their percent composition. However, the purpose of this research was to see if insulin undergoes any conformational changes after its attachment onto the MNPs. This query can be answered by the fact that all of the peaks and valleys are found on locations that are very close to each other within all three curves. This so called 'superimposition' observed in the curves means that no structural or conformational changes took place in the insulin's structure after its attachment onto MNPs. If there were any shifts present within the peaks and valleys on the curves, especially within the 1510 – 1580 (Amide II band) and 1600 – 1700 (Amide I band) wavenumber ranges, which would have meant that somehow insulin's conformation had changed. Since no significant shifts are observed, we can safely say that insulin keeps its native conformation. FTIR spectra are presented in Figure 4-4.

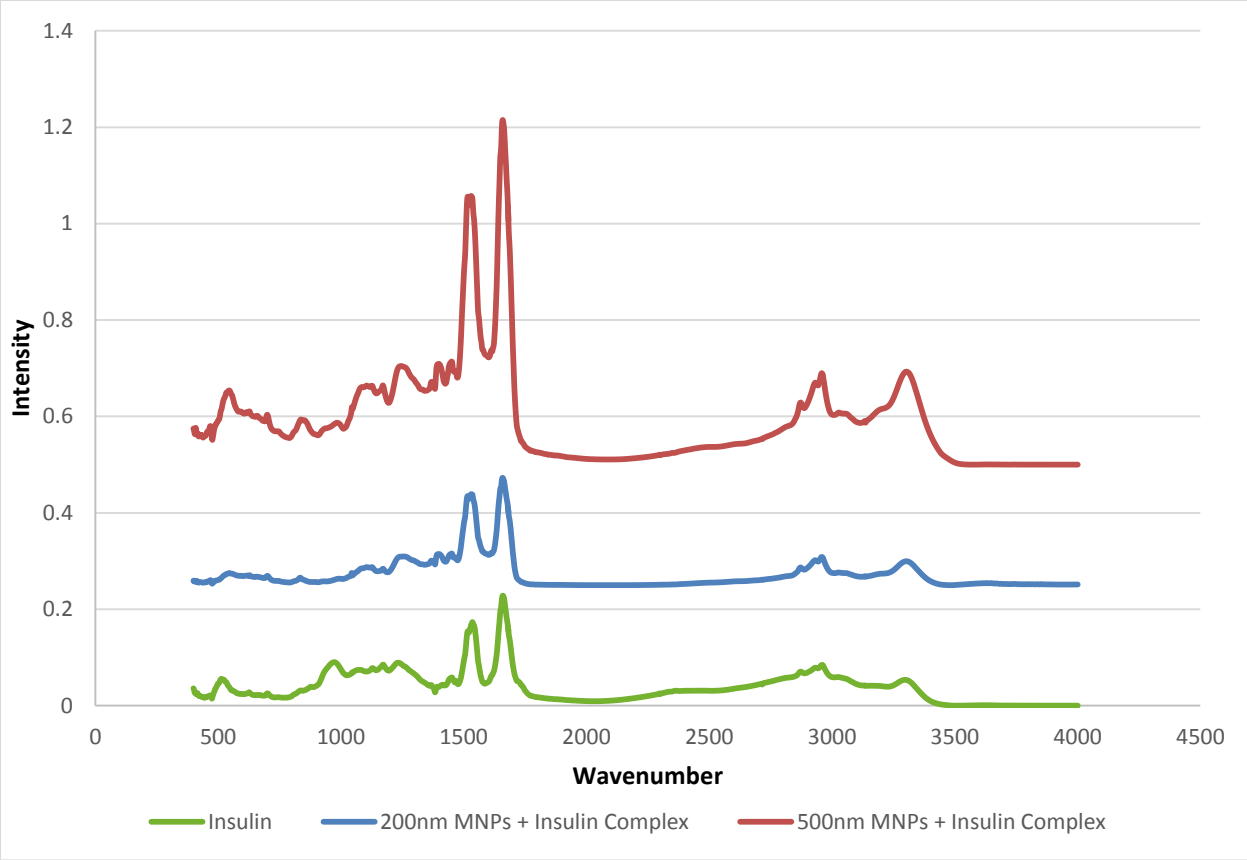

Figure 4-4: FTIR spectra of insulin, 200 nm MNP + Insulin Complex and 500 nm MNP + Insulin Complex.

Further analysis of the amide I band is presented below. Amide I band is found in the range of 1600 – 1700 cm^{-1}. Amide I is observed to be the most intense absorption band in proteins and is directly related to the protein backbone conformation. It mainly shows the stretching vibrations of the carbon-oxygen double bond (C==O) (70 – 85%) and about 10 – 20% of carbon-nitrogen (C---N) group vibrations (Jabs., n.d). Any significant changes within this band would represent a conformational change in the protein's backbone. As Figure 4-5 below shows the peak for all of the curves occurs at the 1658.98 wavenumber, this means that there was no shift observed within the samples. From this fact it can be concluded that insulin's conformation did not change after its binding onto the MNPs. Achievement of these results further confirms the results from the CD spectrum and refers to Objective 2.

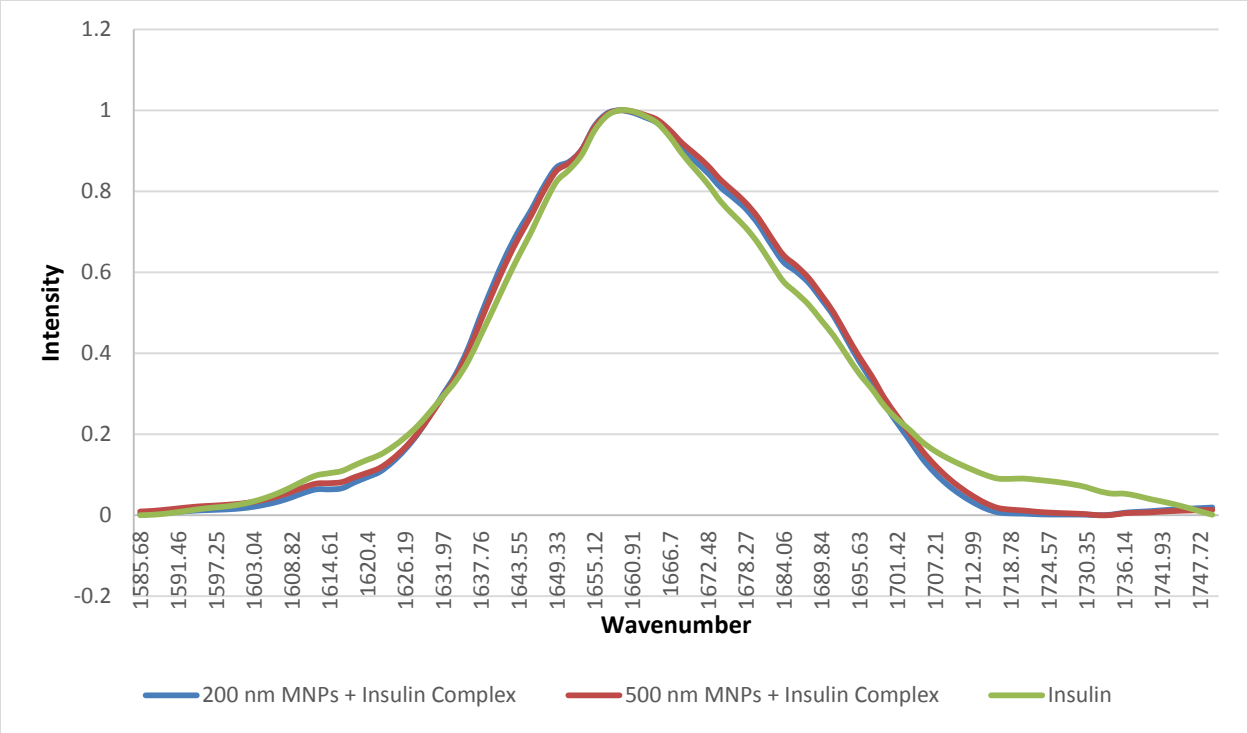

Figure 4-5: Amide I band of insulin, 200 nm MNP + Insulin Complex and 500 nm MNP + Insulin Complex.

Chapter 5: Conclusion and Future Work

5.1 Conclusion

After looking at the limitations of the current diabetes treatment/management strategies an interesting idea was developed, which would help improve the quality of life for diabetic patients and provide a possible solution to the problems associated with the insulin refill of the glucose-responsive implantable devices. This idea can be summarized by the term "Insulin Reuse"; if the patients could use the insulin stored in the implantable devices for multiple cycles it would increase the availability of insulin and would subsequently decrease the requirement for multiple/frequent refills. This thesis looked at possible options to achieve this. The approach using magnetic nanoparticles was chosen as most viable. This research work was focused on the first step in the bigger picture idea of insulin reuse. Insulin was attached onto the NH_2 functionalized magnetic nanoparticles reacted with PEGDA and various characterization experiments were run to analyze the conformation of insulin. The purpose of these characterization experiments was to confirm that insulin does not undergo any conformational changes after its attachment onto MNPs.

The results presented above show in detail that insulin's conformation was not affected by its attachment onto MNPs. Along with confirming the presence of insulin in the solutions, the fluorescence and absorbance spectra confirmed that second part of *Objective 1* of this research was successfully achieved. The presence of insulin in the solution after dialysis could only have resulted if insulin was able to bind onto the MNPs. Detailed analysis of immunoprecipitation and the use of MNPs in literature review provides information regarding the first part of *Objective 1*. The CD and FTIR spectra used for further investigation provided details regarding the

conformation of insulin before and after its attachment onto the MNPs. The results from both of these spectra confirmed that *Objective 2* of the research was successfully achieved as well. In achieving both of these objectives, the *Research Question* was also answered: Insulin can be attached onto the MNPs and its conformation will not be affected by the attachment.

This study provided us with a new avenue of research that can be explored much further. As mentioned above this research has proven that insulin can be successfully attached onto the magnetic nanoparticles without any changes in its conformation. This information can be incorporated into various other ongoing studies on diabetes treatment and development of glucose-responsive implantable devices. The big picture idea discussed in this research has the potential to extend the bioavailability of insulin in these devices significantly. As suggested by the big picture idea, if insulin can be reused for multiple cycles, the maximum functional life of these devices can be increased. This provides a potential solution to a major limitation of such devices: maximum functional life of glucose-responsive implantable devices is too short.

5.2 Limitations

A. Our group only had access to two different sized nanoparticles (200 and 500 nm). The results obtained from these two sizes provide us with enough information to answer both of our objectives and can potentially be generalized over a wider range of sizes. It would have been desirable to have an even wider range of nanoparticles available to perform the experiments.
B. This study was limited to in vitro testing. Although the in vitro testing is a stepping stone in a wider scheme of things, it limited the study to the conformational aspect and could not provide information regarding the functionality of insulin after the attachment.

5.3 Future Direction

This research has shown that insulin's structure remains free of any conformational changes after its attachment onto MNPs. The next logical step would be to run some experiments that would test the biological activity of this insulin + MNP complex. These experiments could involve the usage of an even more diverse size range of nanoparticles. This would allow the research group to identify an ideal sized magnetic nanoparticle that should be used going further. If the insulin proves to be functional after those tests, the research could move onto the next steps in the bigger picture – insulin reuse. A more detailed literature review can be performed to shed light on the physiological requirements that the insulin-MNP complex would have to abide by in order to be used in vivo. Any new information that comes to light because of this literature review can be incorporated into the MNP and hence the insulin-MNP complex design. Besides the biological and physiological considerations, an analysis of design elements can be conducted with respect to the engineering impacts of such a complex.

Also, an economic analysis can be performed to assess the economic feasibility of the work, resources and research that is going to be required in making the big picture a reality. As the insulin-MNP complex created is going to be used in a glucose-responsive implantable device, the requirements and needs of such a device are also worthy of consideration. The research group, going forward, can collaborate with another research group that is working to develop a glucose-responsive implantable device and obtain important insights into the design elements. Potentially, an in vivo study/experiment can be performed in animals.

Appendix: CD Spectroscopy Error Margin

	180-260	185-260	190-260	195-260	200-260	205-260	210-260
NNET_13	4.32%	4.37%	4.35%	4.51%	4.63%	4.84%	4.91%
NNET_23	4.87%	5.06%	5.03%	5.16%	5.38%	5.47%	5.44%
NNET_33	3.98%	5.92%	5.57%	6.20%	6.45%	6.39%	6.60%

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